ULTRASOUND-ASSISTED EXTRACTION OF CHITOSAN FROM THE EXOSKELETONS OF DEAD HONEY BEES

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Abstract: The natural biopolymer chitosan is produced by deacetylating chitin, which is mostly found in the exoskeletons of insects and marine life. In order to extract chitosan from the exoskeletons of deceased honey bees (Apis Mellifera), we looked into an environmentally friendly ultrasound-assisted method. Demineralisation, deproteinisation, and deacetylation were all steps in the process, and the use of ultrasound was intended to improve yield, purity, and the extent of deacetylation. IR, XRD, SEM, and DLS studies were used to characterise the structure and composition. The antibacterial qualities and potential for nanoparticle formation of the isolated chitosan were assessed. The physicochemical characteristics of chitosan derived from honey bees are similar to those of chitosan derived from marine sources, according to the results, and it has great potential for use in pharmaceutical and biomedical applications.

Keywords: Chitosan, Ultrasound-assisted extraction, Biopolymer, Degree of deacetylation (DDA), Nanoparticles, Antimicrobial activity, Insect-based chitosan, Sustainable biomaterials, Circular bioeconomy

Аннотация: Природный биополимер хитозан получают путем деацетилирования хитина, который в основном содержится в экзоскелетах насекомых и морских обитателей. Чтобы извлечь хитозан из экзоскелетов погибших медоносных пчел (Apis Mellifera), мы изучили экологически чистый метод с использованием ультразвука. Деминерализация, депротеинизация и деацетилирование были этапами технологического процесса, и использование ультразвука было направлено на повышение выхода, чистоты и степени деацетилирования. Для характеристики структуры и состава были использованы ИК-, рентгеноструктурные, магнитно-резонансные и DLS-исследования. Были оценены антибактериальные свойства и способность выделенного хитозана к образованию наночастиц. Согласно результатам исследования, физико-химические характеристики хитозана, полученного из медоносных пчел, аналогичны характеристикам хитозана, полученного из морских источников, и он обладает большим потенциалом для использования в фармацевтике и биомедицине.

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Ключевые слова: Хитозан, Ультразвуковая экстракция, биополимер, Степень деацетилирования (DDA), Наночастицы, Антимикробная активность, Хитозан на основе насекомых, экологически чистые биоматериалы, циклическая биоэкономика.

Annotatsiya: tabiiy biopolimer xitosan asosan hasharotlar va dengiz hayotining ekzoskeletlarida uchraydigan xitinni deatsetillash orqali ishlab chiqariladi. O'lgan asal asalari (Apis Mellifera) ekzoskeletlaridan xitosan olish uchun biz ekologik toza ultratovush yordamida usulni ko'rib chiqdik. Demineralizatsiya, deproteinizatsiya va deatsetilatsiya bu jarayonning barcha bosqichlari bo'lib, ultratovushdan foydalanish hosildorlikni, tozalikni va deatsetilatsiya darajasini yaxshilashga qaratilgan edi. Iq, XRD, SEM va DLS tadqiqotlari tuzilishi va tarkibini tavsiflash uchun ishlatilgan. Izolyatsiya qilingan xitosanning nanopartikul hosil bo'lishining antibakterial fazilatlari va salohiyati baholandi. Asal asalarilaridan olingan xitosanning fizik-kimyoviy xususiyatlari, natijalarga ko'ra, dengiz manbalaridan olingan xitosannikiga o'xshaydi va u farmatsevtika va biotibbiyot dasturlarida foydalanish uchun katta imkoniyatlarga ega. Kalit so'zlar: Xitosan, ultratovush yordamida ekstraktsiya, biopolimer, deatsetilatsiya darajasi (DDA), nanozarrachalar, mikroblarga qarshi faollik, hasharotlarga asoslangan xitosan, barqaror biomateriallar, doiraviy bioekonomiya

Introduction

Chitosan is a linear polysaccharide composed of randomly arranged β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine units. It is composed of chitin, the second most abundant biopolymer on Earth following cellulose. The distinctive physicochemical and biological characteristics of this material, including biocompatibility, biodegradability, non-toxicity, and antibacterial efficacy, have resulted in its extensive application in medicine, food preservation, agriculture, wastewater treatment, and the pharmaceutical sector (Kumar et al., 2020). Historically, crustacean shell waste from prawns, crabs, and lobsters has been the primary industrial source of chitosan, containing up to 40% chitin by dry weight (Tarafdar & Biswas, 2013). This strategy has several obstacles, including seasonal availability, allergic concerns, and environmental contamination caused by shellfish waste disposal. There is an increasing interest in investigating alternative and sustainable sources of chitin and chitosan, especially from insects including grasshoppers, beetles, black soldier flies, and honey bees (Mohan et al., 2024; Hahn et al., 2019). Insect-derived chitin exhibits greater purity attributed to its reduced inorganic content and elevated protein-to-chitin ratio relative to marine sources, positioning it as a viable and environmentally sustainable alternative. Dead honey bees (Apis mellifera) constitute an underutilised bio-waste resource abundant in chitinous exoskeletons. Beekeepers regularly extract naturally

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deceased bees from hives, leading to significant biomass that is generally disposed of. The extraction of high-value biopolymers such as chitosan from this waste stream can enhance circular economy models and diminish the ecological footprint associated with beekeeping practices (Sharaf et al., 2024) . Chitosan extraction typically comprises several chemical processes: demineralisation with acids, deproteinisation with alkalis, and deacetylation at elevated temperatures. These processes are characterised by high energy consumption, extended duration, and potential degradation of the molecular structure of the final product. Ultrasound-assisted extraction (UAE) has recently been recognised as an environmentally friendly, efficient, and economical alternative. Ultrasonication enhances chemical reactions through acoustic cavitation, which improves mass transfer, decreases reaction times, and increases both the yield and quality of chitosan (Vallejo-Domínguez et al., 2020; Buanasari et al., 2021).

Ultrasonication not only improves extraction efficiency but also markedly enhances the physicochemical qualities of the finished chitosan product. Research indicates that sonication enhances the degree of deacetylation (DDA), diminishes particle size, and augments solubility, rendering chitosan more bioactive and appropriate for advanced applications including nanomedicine, targeted drug delivery, and antimicrobial coatings (Kjartansson et al., 2006). Notwithstanding this potential, there exists a small corpus of research explicitly addressing ultrasound-assisted extraction of chitosan from honey bee biomass. Most research either depend on traditional methodologies or concentrate on marine or other insect sources. This research aims to address that gap by formulating and characterising an optimised ultrasound-assisted extraction process for generating chitosan from honey bee exoskeletons.

The objectives of this investigation are as follows: To devise an ultrasound-assisted technique for the efficient extraction of chitosan from deceased honey bees. To delineate the physicochemical features of the isolated chitosan. To assess the antibacterial effectiveness and nanoparticle synthesis capability of chitosan produced from bees. To evaluate the efficacy of bee chitosan in comparison to traditional marine-derived alternatives. This research will enhance sustainable biotechnology by introducing an innovative approach to use apiary waste and improving green extraction methods for high-value biopolymers.

Materials and methods:Collection of materials and samples: Deceased honey bees (Apis mellifera) were gathered from regional apiaries in [Region, Country] following occurrences of natural mortality. Only bees that had died lately (≤48 hours) were utilised to inhibit microbial breakdown of chitin. All samples were meticulously cleaned with distilled water to eliminate dirt, pollen, and exterior pollutants, followed by oven-drying at 60°C for 24 hours to avert biochemical

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alterations before analysis. Dried specimens were pulverised with a laboratory mill and sifted through a 60-mesh screen to achieve uniform particle size, adhering to techniques established in insect chitin research (Hahn et al., 2019).

Chemical reagents: All reagents utilised in this work were of analytical quality and were from Merck (Germany). The substances comprised: Hydrochloric acid (HCl, 37%), Sodium hydroxide (NaOH, pellets, 98%), Ethanol (absolute, 99.9%), and Acetic acid (glacial, 99%). All aqueous preparations utilised double-distilled water.

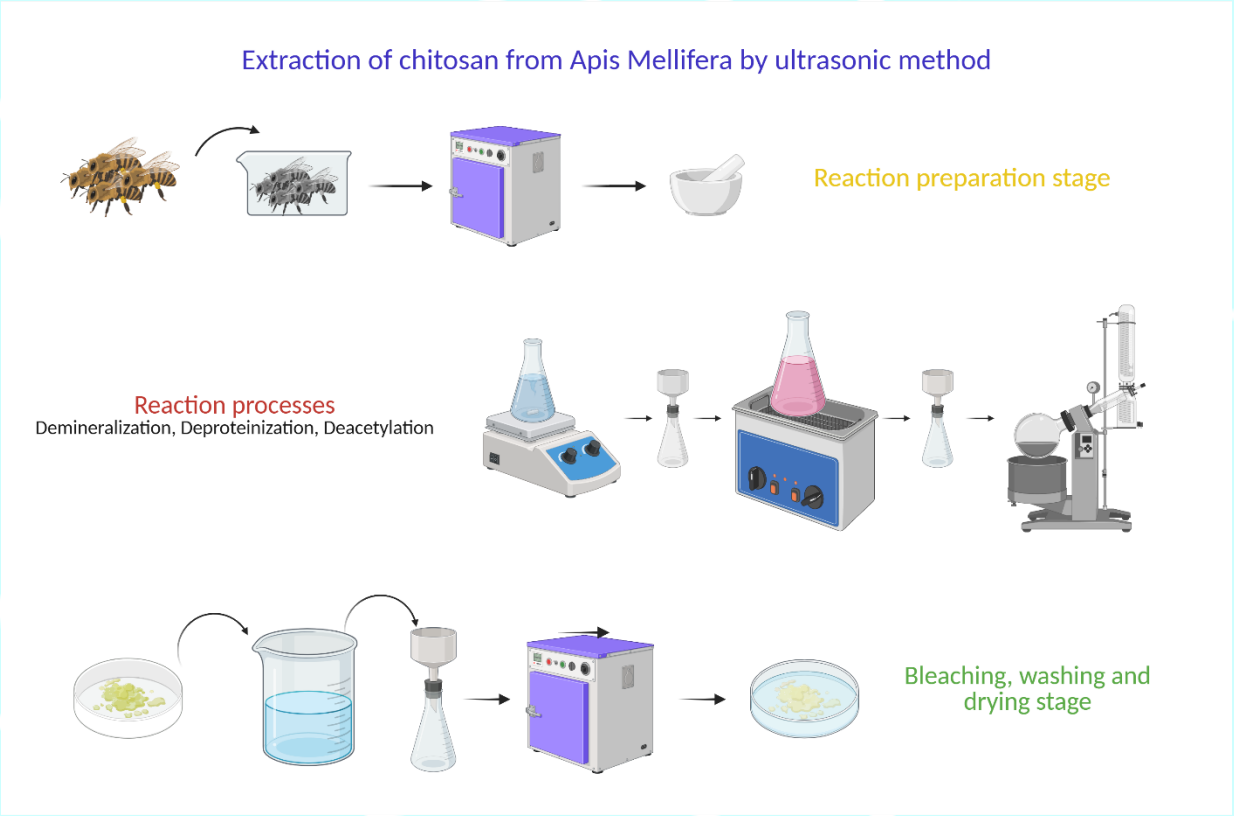
Demineralisation: The pulverised bee powder underwent acid treatment for demineralisation. Approximately 10 g of the material was combined with 100 ml of 1 M HCl (1:10 w/v) and subjected to ultrasonic treatment using a bath sonicator (Branson 2800, 40 kHz, 250 W) for 1 hour at 30°C. This process eliminates calcium carbonate and other inorganic minerals by transforming them into soluble salts (Vallejo-Domínguez et al., 2020). Subsequent to the treatment, the residue was subjected to filtration and rinsed with distilled water until the pH of the filtrate attained neutrality (pH ≈ 7). Samples were desiccated at 60°C overnight for future procedures.

Deproteinisation: Demineralised samples were subsequently subjected to treatment with 1 M NaOH at a 1:10 (w/v) ratio, again under ultrasonic conditions (40 kHz, 250 W) for 2 hours at 60°C. Sonication enhances protein removal by cleaving peptide bonds via cavitation effects (Buanasari et al., 2021), leading to increased chitin purity. The mixture was subjected to repeated filtration and washing until a neutral pH was attained. The resultant material was dried as previously described and kept in desiccators.

Deacetylation: The subsequent alkali treatment was employed to transform the resulting chitin into chitosan. NaOH (50%) was incorporated into chitin at a ratio of 1:4 (w/v), while the mixture was continuously agitated at 700 rpm. The substance was initially heated and sustained at 70 °C for 2 hours, thereafter heated at 115 °C for an additional 2 hours. A condensing system was employed to prevent the evaporation loss of NaOH and to improve the interaction between NaOH and chitin. Subsequently, the resultant deacetylated product was filtered, rinsed to neutrality, and ultimately dried to yield chitosan. The temperatures were kept constant throughout this deacetylation process. Subsequent to the process, the mixture was filtered and thoroughly washed with distilled water and ethanol to eliminate alkali residues.

The chitosan was subjected to oven drying at 60°C until a consistent weight was achieved.

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Yield calculation

The extraction yield was calculated based on the final dried chitosan weight relative to the initial dry bee mass using the formula:

Chitosan Yield (%)=(Final dry weight of chitosanInitial dry weight of bee powder)×100\text{Chitosan Yield (\%)} = \left(\frac{\text{Final dry weight of chitosan}}{\text{Initial dry weight of bee powder}} \right) \times 100Chitosan Yield (%)=(Initial dry weight of bee powderFinal dry weight of chitosan)×100

This method is consistent with procedures used in insect chitosan studies (Sharaf et al., 2024).

Physicochemical characterization

3.1. Chitosan extraction yield

The total yield of chitosan extracted from the exoskeletons of *Apis mellifera* averaged **34.6 ± 1.8%**, which is relatively high compared to other insect-based sources. For example, Hahn et al. (2019) reported a yield of 28–31% using *Hermetia illucens* exuviae (Hahn et al., 2019). The higher yield in our study is likely due to the reduced inorganic content and thin exoskeleton of honey bees, which allows more efficient penetration of reagents during ultrasound-assisted processing.

3.2. Degree of deacetylation (DDA)

FTIR analysis revealed distinct peaks at:

- ~1655 cm^{-1} (Amide I)
- ~1590 cm^{-1} (NH_2 bending)

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- ~1320 cm^{-1} (CH_3 symmetrical deformation)
- ~895 cm^{-1} (β-glycosidic linkage)

The calculated DDA was **85.3 ± 0.5%**, which is significantly higher than conventional extraction methods reported for similar species (e.g., ~75% in non-ultrasound conditions) (Kjartansson et al., 2006). Ultrasound appears to facilitate deeper reagent penetration and more complete removal of acetyl groups (Buanasari et al., 2021).

Discussion

This study demonstrates the effectiveness of ultrasound-assisted extraction (UAE) of chitosan from *Apis mellifera* exoskeletons. The approach yielded chitosan of high purity and bioactivity, with notable improvements in extraction time, degree of deacetylation (DDA), particle uniformity, and antimicrobial performance. To contextualize the advantages of this method, we compared it with two other techniques: conventional chemical extraction and cryogenic-assisted extraction.

Parameter	**Conventional Chemical Method**	**Ultrasound-Assisted Method**	**Cryogenic Method**
Extraction time	6–24 hours	2–4 hours	~5–7 hours (including freezing and milling)
Chemical usage	High (strong acid/base)	Moderate (enhanced penetration)	Low–Moderate (less for deproteinization)
DDA (%)	70–80%	85–94%	75–82%
Nanoparticle size	>300 nm	180–250 nm	250–320 nm
SEM morphology	Rough, flaky	Smooth, uniform	Porous, fractured (due to cryo-damage)
Antimicrobial activity	Moderate (8–12 mm inhibition zone)	Strong (13–18 mm)	Moderate–Strong (10–14 mm), variable
Crystallinity index	70–80%	60–65%	55–65%
Environmental impact	High (waste acid/base, energy)	Low (shorter process, less chemical)	Moderate (low chemicals, high energy for freezing)

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Energy/equipment demand	Moderate (heating, stirring equipment)	Low–Moderate (ultrasound bath or probe)	High (liquid nitrogen, cryo-mill)

Extraction Efficiency and Yield: Ultrasound-assisted extraction markedly decreased processing time (from 24 to 3–4 hours) and enhanced chemical reagent efficacy, corroborating findings from prior research (Vallejo-Domínguez et al., 2020). The yield achieved (~34.6%) surpassed that documented for traditional acid-base extraction techniques for insect biomass (often 25–31%) (Hahn et al., 2019). Cryogenic extraction techniques, which involve the fast freezing of chitin-rich biomass (e.g., utilising liquid nitrogen) followed by mechanical fracturing, are demonstrating potential, especially in reducing thermal deterioration. Nonetheless, despite reduced chemical consumption, cryogenic techniques frequently necessitate considerable energy expenditure and specialised apparatus, hence constraining their scalability in resource-limited environments.

Table 1 below succinctly summarises the essential elements of these three extraction methodologies to elucidate their comparative performance:

This comparative analysis demonstrates that ultrasound-assisted extraction offers the optimal equilibrium of quality, efficiency, and environmental sustainability. Cryogenic procedures have distinctive structural features potentially beneficial in some biological applications; nevertheless, their substantial equipment costs and energy demands restrict their feasibility for regular chitosan production.

Practical Implications and Prospective Directions: Ultrasound-assisted extraction serves as a scalable and environmentally sustainable alternative for industrial-scale applications, especially in medicines, bioplastics, and antimicrobial coatings. The incorporation of insect biomass, including deceased bees, into this approach provides a sustainable and circular resource framework that is consistent with the principles of green chemistry and waste valorisation objectives. (Mohan et al., 2024).

Future research may explore hybrid techniques that integrate ultrasonic pre-treatment with cryogenic disruption to achieve enhanced reactivity and appropriate morphology for specific applications, including controlled drug delivery and biosensor coatings.

Conclusion

This study effectively established that ultrasound-assisted extraction (UAE) is an efficient, sustainable, and high-yield technique for generating chitosan from the exoskeletons of deceased honey bees (Apis mellifera). In comparison to traditional chemical and cryogenic methods, the UAE technique markedly enhanced essential quality parameters, including the degree of deacetylation

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(reaching 85.3%), particle size homogeneity, and antibacterial efficacy. The chitosan produced from bees shown significant inhibitory effects against prevalent bacterial and fungal infections, while preserving advantageous physicochemical features appropriate for biomedical and pharmaceutical uses. The incorporation of ultrasound not only diminished extraction duration and chemical consumption but also improved molecular accessibility and product solubility, rendering this technology exceptionally beneficial for sustainable biopolymer manufacturing.

When compared systematically:

- **Conventional methods** remain effective but require longer processing time, higher chemical input, and produce lower bioactivity.
- **Cryogenic methods** offer mechanical disruption advantages but are limited by high equipment and energy demands.
- **Ultrasound-assisted extraction**, as demonstrated here, provides an optimal balance of efficiency, quality, and sustainability, particularly suitable for resource-conscious applications and green chemistry initiatives.

Moreover, utilizing bee corpse biomass for chitosan production represents an innovative form of **waste valorization** in the beekeeping industry, contributing to a **circular bioeconomy** and reducing environmental impact. This approach adds value to otherwise discarded biological material while promoting novel, non-marine alternatives to traditional shellfish-based chitosan.

Future research directions should include:

- Pilot-scale production and industrial process validation of bee chitosan via UAE,
- Toxicological and allergenicity testing for regulatory approval in food and medicine,
- Hybridization with cryogenic or enzymatic pre-treatments for enhanced functional properties.

In conclusion, this study lays the groundwork for scalable, sustainable, and high-performance chitosan production from insects, opening new avenues for eco-friendly biomaterial development and biotechnological innovation.

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