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Comparative Study on Cryogenic and Conventional Chitosan Production from *Apis Mellifera* Dead Honey Bees: Process Optimization, Characterization and Environmental Impacts

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Abstract The study compares the production of chitosan from *Apis mellifera* (dead honey bees) using cryogenic and conventional methods, finding that the cryogenic method saves time, energy and water while producing chitosan with similar physicochemical properties. In this research work, it was found that obtaining chitosan cryogenic method cost and time are saved to conventional method. The study also uses various analytical techniques such as SEM, TGA and DTG analysis to characteriza the produced chitosan. The obtaining chitosans were applied in the dyeing process.

Keywords *Apis mellifera* dead bees, Chitin, Chitosan, Biomaterials, Pre-deacetylation steps, Chemical extraction, SEM and TGA analysis

1. Introduction

Chitosan is a useful biopolymer obtained by alkaline deacetylation of chitin. Generally, chitin is converted into chitosan with various degrees of deacetylation (DD) and molecular weights (Mw) depending upon the purpose of chitosan utilization. Chitosan differs from chitin in that it is soluble in mild acidic medium. The cationic form of chitosan in acidic solution plays a role in not only governing its solubility but also acting as an active site. The applications of chitosan for improving dyeability of cotton/polyester blended fabric have been widely studied. In the textile area, the higher the active site of chitosan favors the higher the dye adsorption (including natural dye) as well as film formation on fiber surface. Chitosan film on fabric surface is not desirable since it causes the problem of fabric stiffness (poor handling). Fortunately, these effects could be adjusted by the usage of chitosan's proper molecular weight. Normally, chitosan with various molecular weights could be achieved by depolymerization techniques [1,2]. Chitin is a polysaccharide made from N-acetyl-D-glucosamine units which is found mainly in crustacean shells, insects, and microorganisms such as fungi, algae, and yeasts [3]. Extracted chitin is classified as α, β, and γ-chitin according to anti-parallel, parallel, and alternated alignments of the polymer chains (as a combination of α and β structures), respectively. α-chitin is usually obtained from the crustacean exoskeletons, specifically from shrimps and crabs; β-chitin can be isolated from squid pens; and -chitin is obtained from fungi and yeast [4]. Crustacean shells and fungal mycelia are the industrial resources of chitin [3]; [5]. In Uzbekistan, the chitin shells of dead honeybees has also been recognized as a valuable source for the extraction of chitin and its derivative, chitosan, which are extensively utilized in diverse scientific and industrial fields [6,7,8,9].

2. Chitin Sources and Obtaining Methods and Applications

α-chitin is the most abundant and stable polymorph which is more common than the β-type, it has an 80% crystallinity index [10]. Arrangement of microfibrils in a-chitin is strongly fixed by hydrogen bonds that limits its water swelling and permeability compared with p-chitin with a lower content of inter-sheet hydrogen bonds. Also, the proportion of chitin in the source material (such as the raw shells) in-fluences the hardness, permeability, and flexibility [11]. Large available quantity of shrimp and crab shells makes them the most popular source of the white, hard, inelastic, nitrogenous polysaccharide, chitin. The main sources of raw material for chitin ptoduction on commercial scale are crustacean exoskeletons, principally crabs and shrimps. And other sources insect shells.

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2.1. Obtaining Methods Chitin/Chitosan

The honey bees contain three major components; 25-35% a-chitin, 40-50 % proteins, 5-10% lipid and 2-5% CaCO3. Pigments and other metal salts are also the minor components [12,13,14]. The chemical isolation process is composed of four steps: removal of inorganic CaCO3 (demineralization), elimination of proteins (deproteinization), removing small amounts of pigments (decoloration/bleaching), and converting chitin to chitosan (deacetylation). Some studies have changed the arrangement of these steps [15]. Also, some pre-treatments such as grinding for enhancing of solution exposure and few post-treatments like purification techniques may be added for increasing the quality of the final product. The process of obtaining chitosan consists of several stages, starting with the collection of seasonally dead honeybees, followed by washing and drying. The cleaned and dried bees were then subjected to stepwise processing under both conventional methods and liquid nitrogen conditions. For both approaches, the demineralization stage was carried out using three different concentrations, after which the samples were dried. The results of the dry mass were compared [16] (table 1).

Demineralization reaction causes decomposition of $CaCO_3$ into water-soluble calcium salts (calcium chloride ($CaCl_2$)) followed by carbon dioxide (CO_2) discharge (equation 1):

$$HCl + CaCO_3 = CaCl_2 + H_2O + CO_2\uparrow$$

The value of CO_2 gas emission depends on the honey bee mineral content and acid penetration quantity into the dead honey bees [17].

Table 1. Comparison of the demineralization stage in cryogen and conventional method

demineralization stage conventional						
HCl conc (%)	Solid to liquid ratio (g/ml)	Temperature (℃)	Duration	Remained minerals in chitin (%)	Consumed HCl/*Apis mellifera* dry weight (mole/g)	Explanation
2 %	1:4	-	1 h	1,02%	0,012	-
4 %	1:6	Room temperature	5 h	0, 4%	0,013-0,002	Constant stirring
6 %	1:8	20	12 h	0,002%	0,01	-
demineralization stage in cryogen						
HCl conc (%)	Solid to liquid ratio (g/ml)	Temperature (℃)	Duration	Remained minerals in chitin (%)	Consumed HCl/*Apis mellifera* dry weight (mole/g)	Explanation
2 %	1:4	-	1 h	1,2%	0,012	-
4 %	1:6	Room temperature	4 h	0,2 %	0,015-0,003	Constant stirring
6 %	1:8	20	12 h	0,02%	0,01	-

Table 2. Comparision of deproteinization procedures for chemical chitin extraction from dead honey bee shells

deproteinization conventional						
NaOH cons (%)	Solid to liquid ratio (g/ml)	Temperature (^{0}C)	Duration	Remained minerals in chitin (%)	Consumed NaOH/*Apis mellifera* dry weight(mole/g)	Explanation
2 %	1:5	60	90 min	-	0,002	Constant stirring
4 %	1:6	80	60 min	0,005	0,012	
6 %	1:10	100	100 min	0,8	0,02	
deproteinization nitrogen environment						
NaOH cons (%)	Solid to liquid ratio (g/ml)	Temperature (^{0}C)	Duration	Remained minerals in chitin (%)	Consumed NaOH/*Apis mellifera* dry weight(mole/g)	Explanation
2 %	1:5	60	90 min	-	0,002	Constant stirring
4 %	1:6	80	60 min	0,003	0,015	
6 %	1:10	100	100 min	0,8	0,02	

Basically, the protein content in biomaterials is the main cause of allergic reactions; therefore, radical deproteinization is very important [11]. The dry mass of the samples, for both methods, underwent the deproteinization stage using three different concentrations of alkali (NaOH) solution at various temperatures and durations. The clarity of the solution determines the duration of the deproteinization process and the number of baths. The average color change indicates the removal of protein and the completion of the process.

The filtered samples, washed with distilled water and neutralized, were placed for drying for 24 hours. The results of the dried residue samples were then compared. (table 2).

A bleaching/depigmentation stage is applied to remove pigments such as melanin and carotenoids. Residual pigments may cause side effects. Therefore, chitin and chitosan must be highly purified [18,19,20]. Table 3. presents the recommended methods for bleaching chitin. No study has compared bleaching methods; therefore, common indicators cannot be proposed. Lower yellowness is considered advantageous.

The simplest modification of chitin is N-deacetylation, in which it is converted into chitosan by removing acetyl groups [19]. Alkaline treatment mainly improves the solubility of chitin due to cleavage of the main chain and disruption of the crystalline structure. Deacetylated chitin is usually obtained through strong hydrolysis at elevated temperatures [20] (Table 4).

Chitin is a polymer consisting of crystalline and amorphous regions. In contrast to the rapidly and highly hydrated amorphous regions, the crystalline regions undergo deacetylation more slowly and do not dissolve. Upon complete amorphization, the crystalline regions are disrupted, leading to an increase in the degree of deacetylation and soluble fractions.

The deacetylation process was carried out using a combination of the traditional method in 35–40% alkali solution at 99–100 ℃ and the nitrogen environment at 85–90 ℃. In addition, the time parameter was 5 hours for the traditional method and 2 hours for the cryogenic method. Chitosan synthesized by the cryogenic method corresponded to the data indicated in the standard of chitosan synthesized by the traditional method from *Apis mellifera* (Table 5).

Table 3. Bleaching/decoloration of chitin/chitosan

Bleaching/decoloration conventional				
H_2O_2 cons (%)	Solid to liquid ratio (g/ml)	Temperature (℃)	Duration	Explanation
6 %	-	50	1 h	Pigment elimination
15 %	1:10	50	30 min	Bleaching constant stirring
30 %	1:6	Room temperature	24 h	bleaching
Bleaching/decoloration cryogen				
H_2O_2 cons (%)	Solid to liquid ratio (g/ml)	Temperature (℃)	Duration	Explanation
6 %		50	1 h	Pigment elimination
15 %	1:10	50	30 min	Bleaching constant stirring
30 %	1:6	Room temperature	24 h	bleaching

Table 4. Deacetylation methods of chitin

Deacetylation conventional						
NaOH cons. (%)	Solid to liquid ratio (g/ml)	Temperature (^{0}C)	Duration	Chitosan properties (%)	Consumed NaOH/*Apis mellifera* dry weight(mole/g)	Explanation
30%	1:6	100	4 h	DD:75	0.10	constant stirring
35%	1:8	90	5 h	DD:80	0.16	
40%	1:10	100	4 h	DD:97	0.38	
Deacetylation nitrogen environment						
NaOH cons. (%)	Solid to liquid ratio (g/ml)	Temperature(^{0}C)	Duration	Chitosan properties (%)	Consumed NaOH/*Apis mellifera* dry weight(mole/g)	Explanation
30%	1:6	100	4 h	DD:85	0.20	constant stirring
35%	1:8	80	3 h	DD:92	0.36	constant stirring
40%	1:10	90	2 h	DD:96	0.42	The samples were treated in liquid nitrogen for 2–3 minutes and subsequently for 2 hours

Table 5. Characterization chitosan *Apis Mellifera* traditional and cryogen

Deacetylation process	Traditional method	cryogen method
Time	5h ±0,1	2 h ±0,1
Total nitrogen content in chitosan, %	7.6 ±0,2	9.5 ±0,3
Degree of deacetylation (DDA), %	83	87
viscosity, η	4.1	3.4
Molecular weight, kDa	240	192

2.2. Chitin /Chitosan Applications

Chitosan functions as a natural biopolymer enhancing plant growth and crop protection. It stimulates seed germination, root development, and stress resistance; acts as a biopesticide and fungicide against fungi, bacteria, and nematodes; improves soil microbial activity and nutrient retention as a soil conditioner; and enhances seed performance when used as a coating. Due to its biocompatibility and hemostatic properties, chitosan accelerates wound healing and is used in surgical dressings. It serves in drug-delivery systems (nanoparticles, hydrogels, films) enabling controlled release, and as a scaffold in tissue engineering for bone, cartilage, and nerve regeneration. Its antimicrobial activity and ability to bind dietary fats and cholesterol support applications in infection control and metabolic regulation. Food Industry-chitosan extends food shelf life through its antimicrobial and antioxidant properties. It is employed as an edible coating on fruits, vegetables, and seafood to reduce spoilage, as a dietary fiber supplement with lipid-binding capacity, and in biodegradable food-packaging films as a sustainable alternative to plastics. Chitosan efficiently adsorbs heavy metals, dyes, oils, and phosphates in wastewater treatment, is utilized in air-filtration materials for toxin removal, forms biodegradable composites replacing synthetic polymers, and has strong hydrocarbon adsorption capacity useful in oil-spill cleanup. In textiles, chitosan improves dye uptake in cotton, wool, and polyester blends; provides durable antimicrobial finishing preventing bacterial and fungal growth; acts as an antistatic and softening agent enhancing comfort; and supports development of medical textiles such as bandages and hospital gowns.

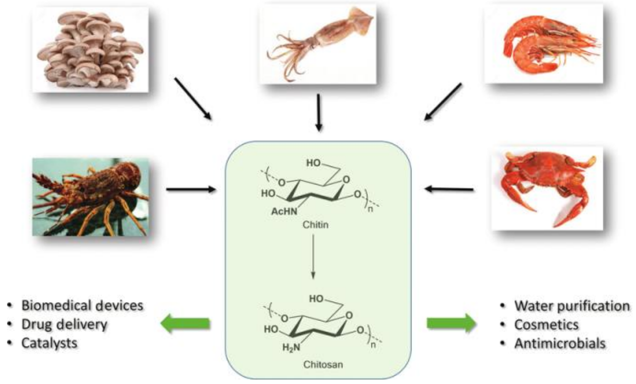


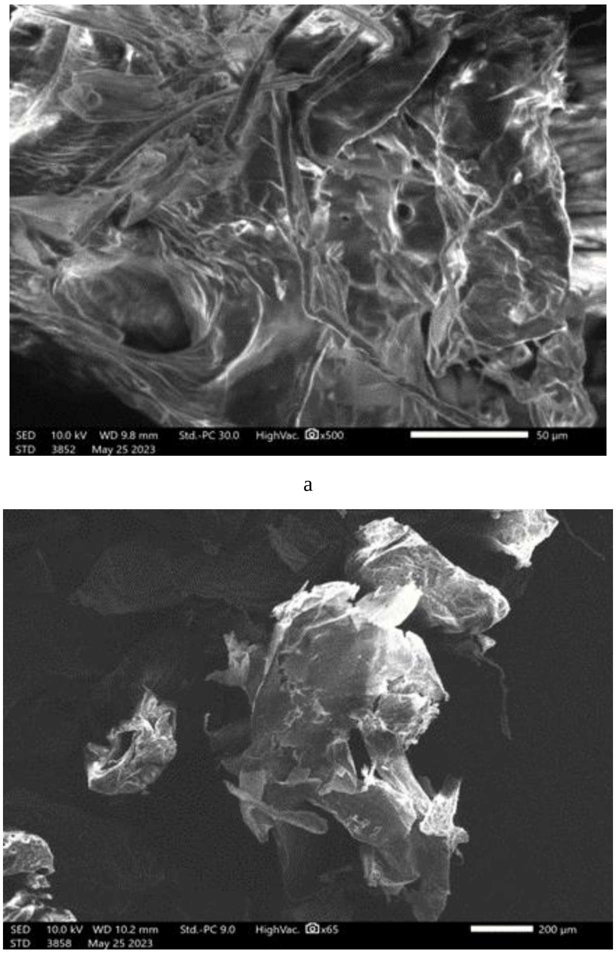
Figure 1. Chitin/chitosan based biomaterial applications [21]

As a film-forming moisturizer, chitosan enhances skin hydration; strengthens and protects hair, improving gloss and reducing breakage; and exhibits antibacterial activity in toothpaste and mouthwash formulations.

Chitosan is applied in enzyme and cell immobilization for biocatalytic processes, in the production of biodegradable bioplastics, as an eco-friendly component in agricultural sprays and coatings, and in nanotechnology for targeted drug delivery and biosensor design.

3. Physicochemical Properties Chitosan

The above figure 2 presents the scanning electron microscopic (SEM) image and elemental analysis of chitosan extracted from *Apis mellifera* (dead honey bees) obtained via the conventional method.

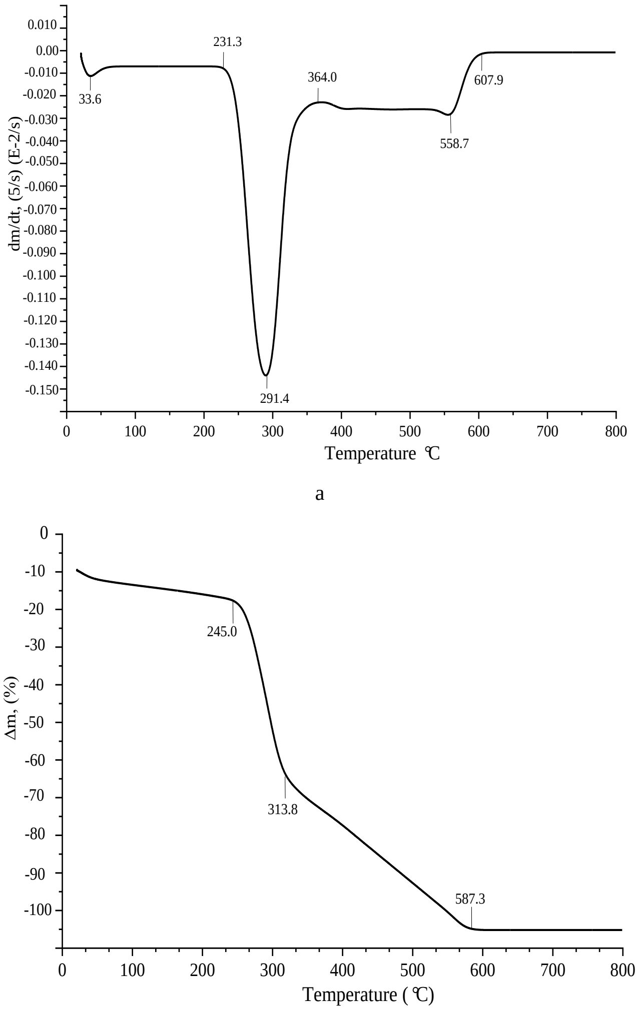


a

b

Figure 2. SEM analysis chitosan: a) traditional b) cryogen

The SEM micrograph clearly reveals the microstructural features of the chitosan surface, showing fibrillar arrangements, pores, and cavities that define the specific morphological characteristics of the sample. In contrast, the quantitative analysis of chitosan synthesized through the cryogenic method indicates that the nitrogen (N_2) content reaches 9.56%, which is notably higher than that of conventionally prepared chitosan. This increase in nitrogen content suggests a higher degree of deacetylation and improved purity of the cryogenically obtained sample.The SEM image of the cryogenically treated chitosan also exhibits a distinctly different morphology. The structure appears more amorphous, with reduced crystallinity compared to the conventional sample. This transformation can be attributed to the cryogenic processing, which minimizes thermal degradation and promotes uniform deacetylation, effectively eliminating structural imperfections formed during heating and drying stages.



a

b

Figure 3. a) DTG b) TG analysis chitosan *Apis mellifera*

Figure 3 (a) the DTG curve of chitosan derived from *Apis mellifera* shows three main thermal degradation regions. The first stage (30–120℃) corresponds to the evaporation of physically adsorbed moisture. The second and most significant stage (230–350℃) represents the main decomposition of the chitosan polymer backbone, including glycosidic bond cleavage and deacetylation of acetyl groups. The final stage (above 350℃) indicates carbonization and formation of stable char residues. Cryogenically processed chitosan exhibited a slightly lower decomposition onset and sharper DTG peak, implying reduced crystallinity, higher deacetylation uniformity, and improved purity compared with conventionally prepared samples. Overall, the thermal behavior confirms that cryogenic treatment enhances the structural homogeneity and thermal stability of chitosan obtained from honey bee biomass. Figure 3 (b) the TG curve of chitosan derived from Apis mellifera shows three distinct weight-loss stages during thermal decomposition. First stage (up to 120℃) - a small mass loss occurs due to the evaporation of physically adsorbed and bound water molecules. This indicates the hydrophilic nature of chitosan. Second stage (245–313℃) - the main degradation phase corresponds to the thermal decomposition of the chitosan polymer backbone, including the cleavage of glycosidic linkages and deacetylation of residual acetyl groups. The maximum degradation rate occurs around 313℃, demonstrating good thermal stability. Third stage (above 500℃, ending near 587℃) - represents carbonization and the formation of a stable char residue. The remaining mass corresponds to carbonaceous and inorganic components. Overall, the TG analysis confirms that *Apis mellifera* based chitosan possesses good thermal stability with gradual degradation, making it suitable for various industrial and biomedical applications.

4. Using Dyeing Process and Environmental Impacts

Chitosan friendly cationization agent. Chitosan increases dye uptake in cotton and polyester–cotton blends by providing additional binding sites. In additional, salt-free dyeing: helps avoid the use of large amounts of electrolytes, making dyeing more eco-friendly and color fastness: improves wash fastness and fixation of reactive, acid, disperse and natural dyes [22].

Chitosan is a biodegradable, renewable, and non-toxic biopolymer derived mainly from chitin found in crustacean shells and insect biomass. Its production and application offer significant environmental advantages compared to petroleum-based polymers. During production, chitosan synthesis involves deproteinization, demineralization, and deacetylation using alkaline and acidic reagents. Although these steps generate some chemical effluents, modern methods - such as microwave-assisted, enzymatic or cryogenic processing-have reduced reagent consumption, energy use and wastewater discharge.

From an ecological perspective, chitosan contributes to sustainable development due to its biodegradability and ability to replace synthetic polymers in packaging, textiles, water treatment, and agriculture. It decomposes naturally into harmless products such as carbon dioxide, water, and nitrogen compounds, minimizing environmental persistence. Moreover, chitosan's ability to adsorb heavy metals, dyes, and pollutants makes it an effective material for environmental remediation, particularly in wastewater treatment. When produced from waste biomass such as shrimp shells or dead honey bees, chitosan also supports waste valorization and circular economy principles.

5. Conclusions

In the research work, chitosan was extracted from local secondary raw materials using two different methods, and the kinetics of the extraction processes were compared. The physicochemical properties of the obtained chitosan samples were analyzed. This biopolymer, which has a wide range of applications, was utilized in the dyeing process of mixed fabrics in the textile industry. Overall, chitosan represents a green alternative polymer that reduces ecological footprint, promotes waste recovery, and supports eco-friendly industrial innovation.

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Declaration

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

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