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COMPARATIVE ANALYSIS OF SOURCES FOR CHITOSAN OBTAINING

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The article highlights the properties and applications of biopolymers such as chitin and chitosan. Traditional sources of chitosan obtaining and problems of their application are reviewed. Alternative, more perspective sources for chitosan obtaining are proposed.

Key words: *chitin, chitosan, marine organisms, mushrooms, microorganisms, insects.*

Introduction. With better appreciation of biopolymers derived from natural organisms, there has been increased interest in their biomedical and industrial applications. Some of important molecules are chitin, chitosan, oligosaccharides and their derivatives, which have attracted significant interest in view of their broad range of applications, including in the biomedical, agricultural, food science, and technological fields, and in various industries [1].

Chitin is a nitrogen-containing polysaccharide which is chemically very similar to cellulose – linear biopolymer of β -D-glucose; only instead of hydroxyl group (OH) next to the second carbon atom on each ring (monomer) of chitin amino group is located, in which one of the two hydrogen atoms is substituted by acetyl group. The both polymers are linear, and in both monomeric units are connected by β -(1-4)-glycosidic bonds (Fig.1) [2].

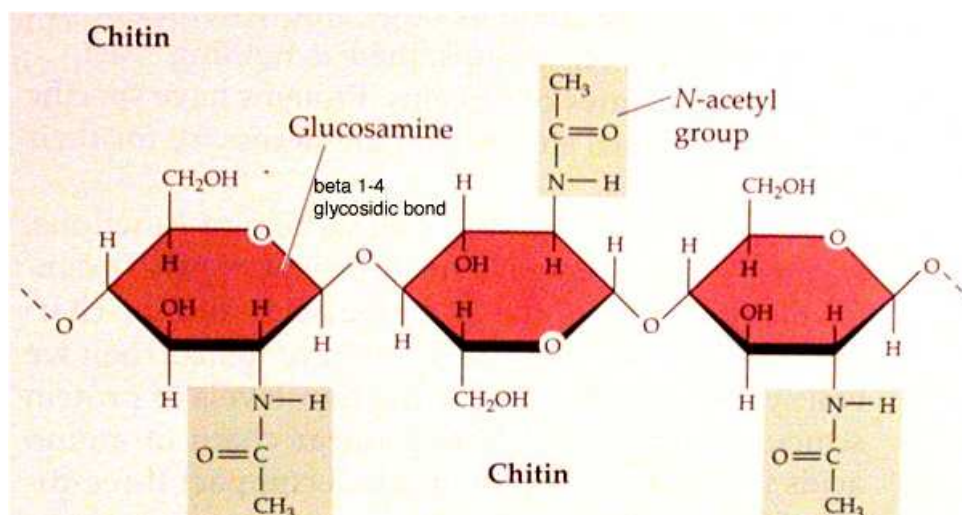


Fig. 1. Structure of chitin molecule

Chitin is one of the most common polysaccharides. It is considered as second after cellulose organic matter by the distribution in the nature [3]. It can be found as supporting materials in many aquatic organisms (in shells of lobsters, crabs, shrimp), terrestrial organisms (scorpions, cuticle of insects), and in some microorganisms and fungi. Each year in the world about 10 gigatons of chitin is produced in living organisms, and then decomposed [1].

In all organisms that produce and use chitin, it is not in pure form but in the combination with other polysaccharides, and is often associated with proteins. In the cell wall of fungi chitin is included into the chitin-glucan complexes; insect cuticle is built from chitin-melanin complexes, chitin of crustaceans' shells is bounded to proteins, so forms chitin-protein complexes.

The deacetylated form of chitin is referred to as chitosan [3, 4].

Chitosan is polysaccharide received from chitin by acetyl group in C2 position removing at alkali solution action (Fig.2) [2, 3, 5]. Chitosan preparations differ by the degree of deacetylation. Unlike chitin, chitosan dissolves in water and diluted organic acids, for example, in aqueous solution of acetic acid. So, this characteristic causes extensive usage of this polysaccharide in different fields of application [6].

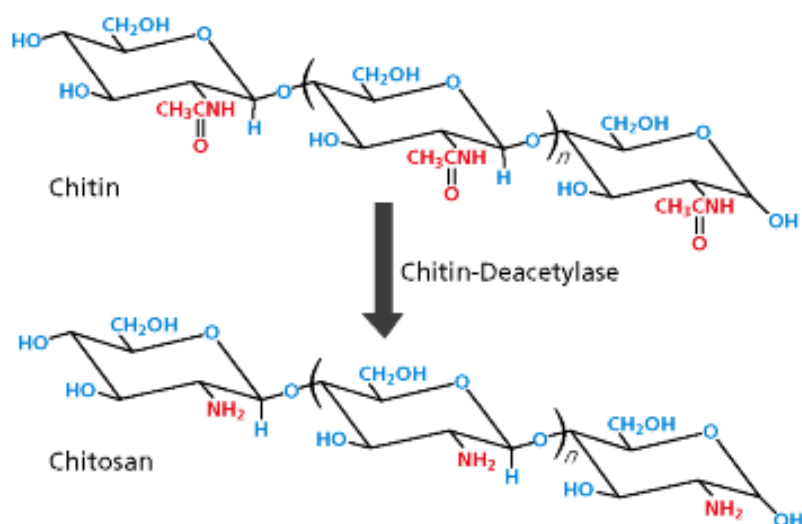


Fig. 2. Obtaining of chitosan from chitin

Chemical modifications of chitosan allow obtaining products with widely varied characteristics, preserving its useful properties. It is referred to as material of the 21st century and this is not accident. More than 15 countries are engaged in researches of chitosan. It has a lot of useful properties making it in some cases indispensable in industry, medicine and agriculture. Now more than 70 practical applications of chitin/chitosan and their modifications are known, mostly in biotechnology, ecology, food and cosmetics industry, medicine, agriculture and veterinary.

While synthetic compounds lose their appeal, natural substances such as chitin and chitosan attract more attention. Diversity of chitosan and its derivatives caused interest to its widespread usage in various industries.

The main fields of chitosan application:

- food industry application – food additives, deposition of starch, preservation food stuffs flavor and taste, manufacture of food films (chitosan films have been developed for the purpose of preventing dampness, reduce the growth of bacteria and increase shelf life of perishable products such as fresh fruits and vegetables. In one of the researches it has been proved that the coating of fresh strawberries by chitosan film increases storage period of berries from one to five days or more);

- numerous medical application;
- biotechnological application (as a matrix for enzymes and microorganisms immobilization);
- ecological application – purification of wastewater and drinking water [7, 8].

Chitosan useful properties:

- high reactivity (participates in reactions of alkylation, carboxylation etc., which allow series of its derivatives with new properties usage);
- radiation resistance (resistance to gamma radiation, so it can be used for the purposes of protection and purification from radionuclides);
- excellent sorption capacity (transient and especially heavy metals: copper, zinc, nickel, cobalt, molybdenum, vanadium, titanium, antimony, ruthenium, strontium);
- selectivity (the ability to separate some metals, iron and copper, nickel and iron, cadmium and nickel and to absorb toxic metals: mercury, cadmium, lead);
- biocompatibility with living tissues (non-allergenic and non-rejection) and non-toxicity, easy excretion from the body;
- biodegradation (the ability to decompose under the action of enzymes);
- bacteriostaticity (the ability to inhibit the growth and reproduction of microorganisms such as bacteria and molds);
- good adhesiveness.

One of the main characteristics of chitosan is the degree of deacetylation (DDA). It influences on the physical, chemical and biological properties of chitosan, such as acid-base and electrostatic characteristics, biodegradability, self aggregation, sorption properties [9].

Nowadays chitin and chitosan are produced commercially from biowastes obtained from aquatic organisms. The main source of raw materials in large-scale industrial obtaining chitosan is shell king crab, shells of shrimp, crayfish, gammarus crayfish. The terrestrial crustaceans, insects, mushrooms and microorganisms (fungi) also may be used as potential chitosan source [10, 11].

Marine organisms are source for chitosan production. The basis for chitosan obtaining from aquatic crustaceans is their waste (shells). As mentioned earlier, chitosan is obtained by removing acetyl groups from chitin as a result of his treatment by alkali solution in harsh conditions. Thus, the stage of deacetylation of chitin is always preceded by the process of its selection from chitin containing material.

Chitin in crustacean cuticles is tightly associated with inorganic salts such as calcium carbonate, proteins, and lipids, including pigments; and as insoluble polymer is not exposed to the release directly from shell. For it obtaining protein and mineral compounds of the shell must be consistently separate, i.e. convert them into soluble state and remove [4].

The isolation of chitin from crustacean shells consists of three coherent steps: deproteination (DP), demineralization (DM), and decolorization (DC); but the order of the first two steps is generally considered irrelevant if protein or pigment recovery is not an objective. Chitin is further deacetylated (DA) to make chitosan or other products for a wide array of applications [12]. Two methods are widely used on an industrial scale for the production of chitosan:

- 1) Chemical extraction;
- 2) Enzymatic (biotechnological) method.

Chemical extraction. Several procedures for the preparation of chitin and chitosan from different shellfish wastes have been developed over the years, some of which form the basis of the chemical processes used for the industrial production of chitin and derivatives [13]. Industrial techniques for chitin and chitosan extraction from different shell waste streams normally rely on harsh chemical processes due to covalent associations with other shell constituents. These methods generate large quantities of hazardous chemical wastes and partial DA of chitin and hydrolysis of the polymer may occur, leading to inconsistent physiological properties in the end products [14].

In general, proteins are first removed (and may be used as animal feed) from ground shells by treating with mild sodium hydroxide or potassium hydroxide

solution at elevated temperature. Alkali concentrations usually between 1 % and 10 % with temperatures ranging from 30 °C to 100 °C, independent of starting material, are most common. Typical reaction times used vary between 30 min and 12 h. For example, the optimal method for the DP of crab shell and shrimp shell waste was reported to be 1 % – 2 % KOH at 90 °C with a shell to alkali solution ratio of 1:20 (w/v). A minimum period of 1 h was needed to extract 90 % of the proteins, with 2 h removing most proteins of the shells [12].

Removal of calcium carbonate and calcium phosphate is accomplished by dissolution with dilute acids. Hydrochloric acid is used most commonly, but other acids can also be used (ethylenediaminetetraacetic acid – EDTA) [15].

Commonly used industrial-scale DP and DM of shellfish wastes produce a brown to brownish-white product that may be bleached using a variety of reagents while removing residual lipids. Most commonly used reagents include ethanol, ether, acetone, sodium hypochlorite, hydrogen peroxide or a combination, chiefly used at ambient temperature.

Chitosan is produced from chitin by deacetylation with highly concentrated (40-50 %) solutions of sodium hydroxide at high temperatures (100-150 °C), exclusive of air, for about an hour [16]. Due to this method chitosan with a degree of deacetylation ranging from 22 to 43 % is received.

Highly deacetylated chitosan usually is not obtained under the conditions mentioned above. Effective deacetylation is attained by intently washing the intermediate product in two or more changes of water during the alkali treatment. The procedure did not significantly degrade the polymer, and recovered a product of molecular weight of about 5×10^5 Da with nearly 100 % deacetylation [17].

A representation of current industrial chitin processes are summarized in Fig. 3 [14].

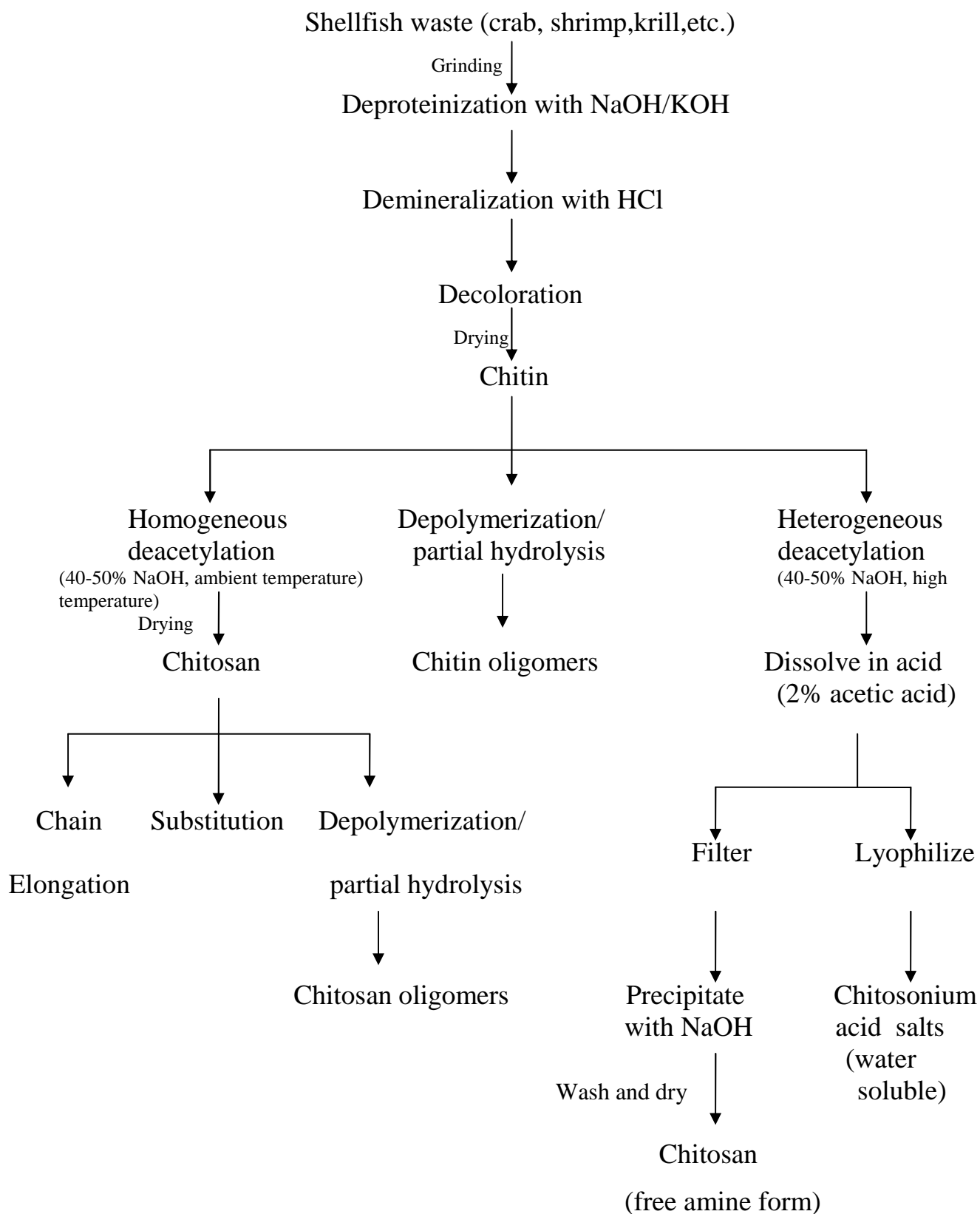


Fig. 3. Industrial processes of chitosan obtaining from shellfish wastes

Enzymatic method. An interesting alternative to chemical methods is the use of biotechnological processes for chitin and chitosan preparation (Fig.4). At this method enzymes of microorganisms are used.

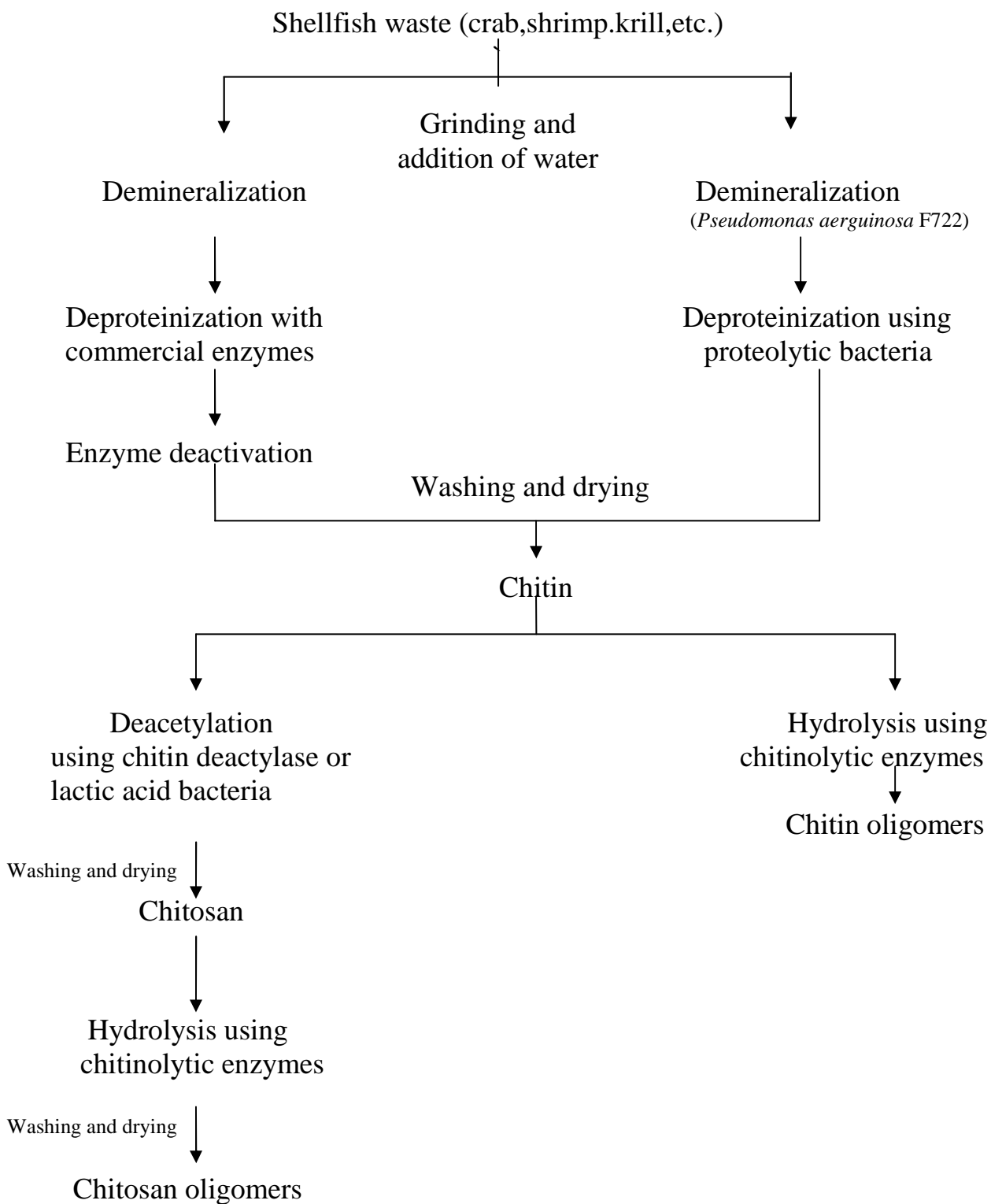


Fig. 4. Industrial processes of chitosan obtaining from shellfish wastes

Proteases can be used for the deproteinization of crustacean shells for the production of chitin or chitosan. Usage of bacterial protease from *Pseudomonas maltophilia* in culture medium with crustacean shell for 24 h cause the protein content remaining in the shells was only about 1 % [18].

Chitin deacetylase from *Colletotrichum lindemuthianum*, *Mucor rouxii*, *Abisidia butleri*, or *Aspergillus nidulans* may convert chitin from shell waste to chitosan. This enzyme shows a pH optimum at 5.5 and is markedly inhibited by acetate.

Usage of *Mucor rouxii* to produce chitosan provides maximum weight after two days of cultures obtained, with a decrease after that period. Molecular weights obtained were over 10^6 Da after two days, with 10 L batch cultures. The deacetylase activity of their purified enzyme showed a pH optimum of 4.5, and the activity at this pH varied markedly with the buffer employed. Tris-HCl buffer solution appeared to be the most suitable for high enzyme activity. In general, unlike the batch systems, molecular weights did not decrease over time in continuous culture systems. In addition, molecular weights were generally higher in the defined culture medium (ammonium sulfate as the nitrogen source, trace of metals and glucose as the carbon source) than in the complex media (nutrient broth, yeast extract, and glucose), although yields of chitosan were significantly lower.

Fermentations with bacteria producing proteolytic and chitinolytic enzymes has been researched as an alternative with varying levels of success [19, 20, 21].

The global amount of chitin annually extracted from waste of aquatic materials is 40.000 tons, and currently a few thousand tons of chitosan is produced per year.

Comparing these two methods attention should be paid to the fact that the chemical method is more beneficial and simple, despite the large numbers of reagents are used. Residual protein, after treatment during enzymatic method, often in high quality remains, and reaction times are significantly increased compared to chemical methods. Also the enzyme costs are furthermore prohibitive, limiting

enzymatic methods in industrial applications unless the process is made more efficient.

During the enzymatic process chitosan with degree of deacetylation ranging from 8.9 to 0.6 % is obtained. This is lower than values obtained by common practices using sodium hydroxide which results in products with a degree of deacetylation ranging from 22 to 43 %. While a preparation of chitin, chitosan by enzymatic means may not be feasible in large-scale industrial production, a combination of chemical and enzymatic reactions may provide a cost effective and environmentally friendly compromise.

Microorganisms are source for chitosan production. Chitosan can be found in fungi, molds, yeasts, some ciliates, and algae.

Microbial synthesis of chitosan has been identified in various organisms including *Mucor rouxii*, *Phycomyces blakesleeanus*, and others. Cell cultures of these organisms have been used for the production of chitosan, and the yields obtained were improved by addition in the culture medium of a chitin source such as *Aspergillus niger*, thus the mechanism of production was attributed to chitin deacetylation. The yields after 96 hr of incubation were of 26.9 % of dry cell wall material, with a chitin source included. Furthermore, the degree of acetylation of chitosan could be reduced to values ranging from 8.9 to 0.6 %.

Generally, the yield and composition of the polysaccharide depends on the used microbial species, age of the producing microbial cells, growth nutrition medium, and ambient conditions. The chitinous compounds content also depends on the type of fermentation and extraction method.

Chitin was first isolated in 1811 from fungi and named fungine. A wide variety of fungi are able to produce chitosan, at contents and yields varying from 0.3 to 12.5% of cell dry weight. Furthermore, they can be cultivated on virtually any substrate, although for sustainability the best substrates are clearly by-products or waste products of other industrial processes, such as rinse washes from distilleries, molasses, whey retentate, or soybean and mungbean residues. Alternatively, chitin and chitosan can be produced directly from waste mycelia of

common industrial fungi, e.g. *Trichoderma reesei* mycelia from cellulase and hemicellulase production or *Penicillium verruculosum*, *Aspergillus niger*, *M. rouxii* or *Rhizopus oryzae* mycelia from antibiotic, citric acid or lactic acid production, respectively. In this context it is worth noting that more than 80,000 tons of waste *Aspergillus niger* mycelium per year is generated from citric acid production alone [9].

Various protocols have been used for chitosan obtaining from fungi in published studies, which can be divided into chemical and enzymatic procedures, as illustrated in Fig. 5 (where the enzymatic method is on the right) [22].

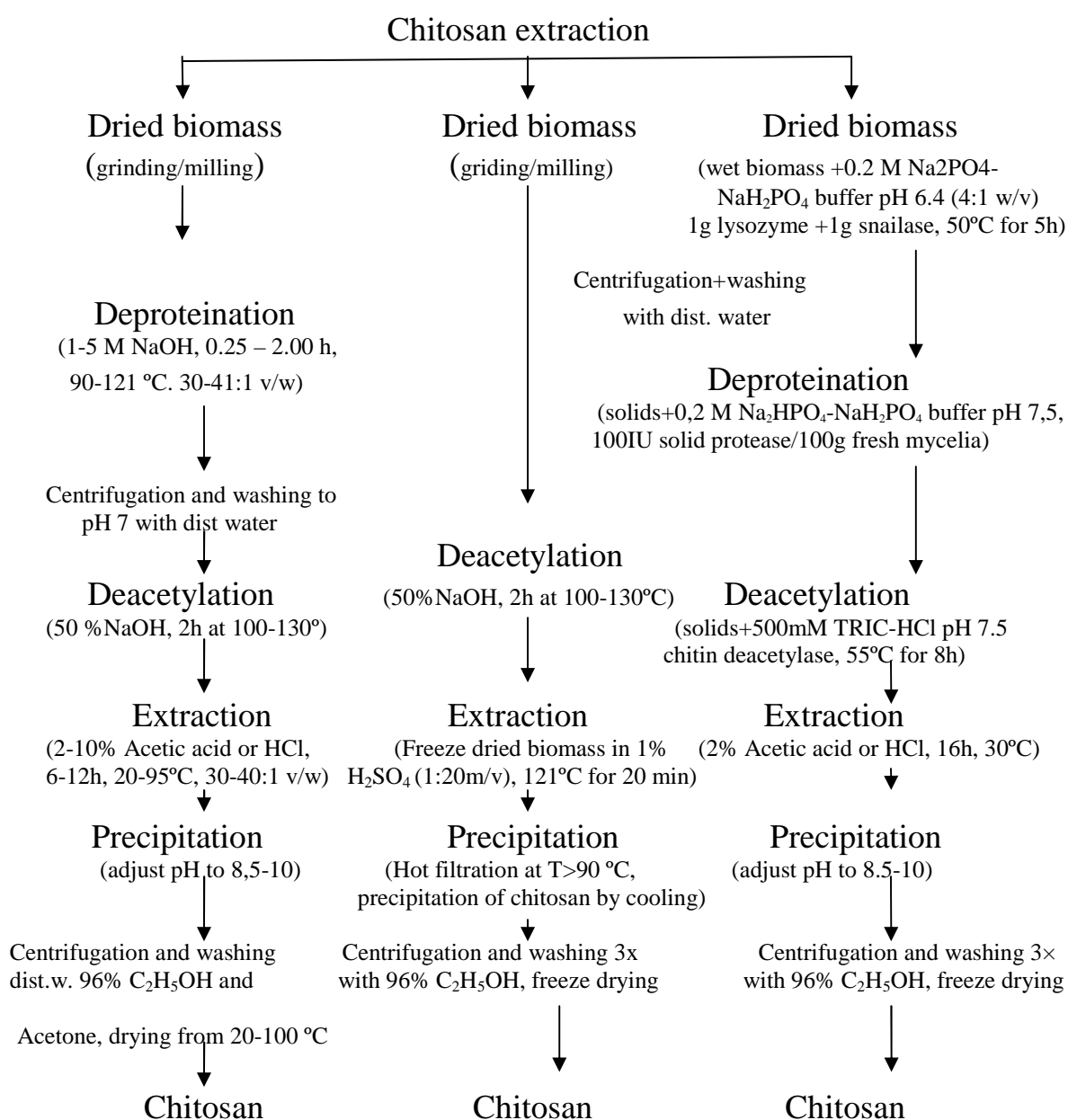


Fig. 5. Different protocols for extracting chitosan from fungi

Known selection methods of chitin are based on the use of 5 % HCl or EDTA (initially used to dissolve or bind the calcium carbonate), then 40 % NaOH at 110 °C to remove the proteins and lipids, with parallel deacetylation of chitin to chitosan. Chitin normally accounts for 14-27 % of the dry weight of shells, 60 - 80 % of it can be converted to chitosan. The fungal chitosan have very low contents of inorganic material. Hence, demineralization is not required. From microorganisms chitosan with deacetylation degree of 6 %-40 % is obtained.

The great advantage of chitosan producing from fungi in compare with crustaceans is that it can be directly extracted from fungal biomass at any time, avoiding seasonal fluctuations. For fungi that only provide chitin in their cell walls an almost identical extraction procedure to that used to extract chitosan from crustaceans must be applied. Hence, it is more economical to use only fungi that already provide the target product. For instance, ready-touse chitosan can be reportedly produced from *Absidia coerulea* very simply by boiling in 25 % NaOH without extracting with hot acetic acid because the chitosan is not intermingled with glucan [23].

Mushrooms are source for chitosan production. The extraction of chitin and chitosan from different species of mushrooms (*Agaricus bisporus*, *Auricularia auriculajudae*, *Lentinula edodes*, *Trametes versicolor*, *Armillaria mellea*, *Pleurotus ostreatus*, *Pleurotus sajor-caju*, and *Pleurotus eryngii*) can be provided. The growth rate of mushroom depends on cultivation conditions such as temperature, moisture content, medium composition and type, and mainly species of the mushroom. The mushroom, *P. sajor-caju* showed highest yield of biomass and *L. edodes* was the lowest when compared with other mushrooms under surface fermentation (Table 1) [24].

The main components of mushroom are water, proteins, chitin, chitosan, and glucans. The extraction of chitin from mushrooms is similar to its extraction from crustacean shells, but the cell wall of mushrooms contains smaller amounts of calcium, so, during obtaining of chitin from mushrooms less concentrated solutions

of acids are used. The yield of extracted chitin and chitosan depends on mushroom species, harvesting time, chitin and chitosan extraction processes and conditions.

The processes and conditions for the extraction of chitin and chitosan from mushroom based on the usage of two methods:

1) used 1 M NaOH at 121 °C for 0.25 h for deproteination and the chitosan was extracted from the collected alkaline insoluble material using 2 % acetic acid at 95 °C for 8–14 h [24];

2) used 1 M NaOH at 40 °C for 15–17 h for deproteination and the chitosan was extracted from the collected alkaline insoluble material using 5 % acetic acid at 90° C for 3 h [25].

Table 1

Yield of chitin and chitosan from mushrooms

Mushroom species	Optimal harvesting time (days)	Yield of biomass (g/L)	Yield of chitin or *chitosan (mg/g of biomass)
<i>L. edodes</i>	12	3	*40
<i>A. bisporus</i>	21	3.5	85
<i>A. auricula-judae</i>	21	6.8	196
<i>T. versicolor</i>	21	3.2	101
<i>A. mellea</i>	21	4.2	131
<i>P. ostreatus</i>	21	6.4	111
<i>P. sajor-caju no</i>	21	10	*12
<i>L. edodes</i>	–	–	*240

*, yield of chitosan (mg/g of biomass).

In general methodic for chitin obtaining is represented on the Fig. 6.

The total yield of chitosan is equal 10–40 mg/g of dried mushroom. For extraction of chitin and chitosan alkaline treatment, decolorization and then deacetylation with concentrated sodium hydroxide solution are used.

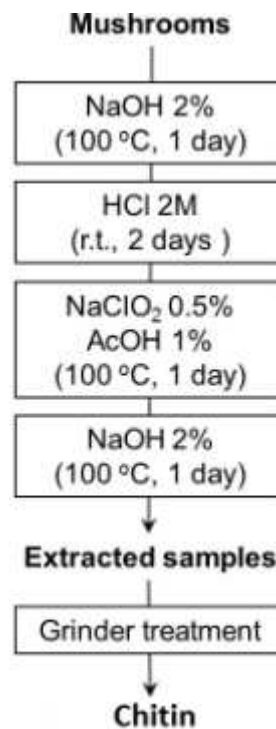


Fig. 6. Preparation procedure of chitin from mushrooms

Mushroom chitosan have a degree of deacetylation of 70 % – 90 % that depends on mushroom species and treatment conditions, and average molecular weight about $1-2 \times 10^5$ Da [26].

So, chitosan which obtained from mushrooms has a higher degree of deacetylation compared with crustaceans and mushrooms chitosan. This greatly improves the chitosan viscosity and solubility in concentrated acids, thereby expanding the scope of chitosan usage.

Insects are source for chitosan production. The composition and biosynthesis of chitin have been studied in insects such as mosquitoes, cockroaches, honeybees, silkworms, *Drosophila melanogaster*, *Extatosoma tiaratum*, *Sipyloidea sipyilus*. These insects contain chitin in their wings and cuticle. Dead bees may be considered a perspective source for chitosan obtaining [27].

The procedure for extraction of chitin and chitosan from the cuticle of insects is similar to that of crustacean sources. The scheme of chitosan obtaining procedure from silkworm pupa and cover of bees is shown in Table 2 (a, b).

Table 2

a)

Procedure for chitosan extraction and yield of chitosan from insects

Species	Treatment Conditions						
	Deproteination			Demineralization			Yield of Chitin (%)
	NaOH Conc. (M)	Temp. (°C)	Time (h)	HCl Conc. (M)	Temp. (°C)	Time (h)	
Silkworm pupa	1	100	42	2	25	96	33
Cover of bees	1	40	2	—	—	—	25

b)

Species	Treatment Conditions						
	Decolorization			Deacetylation			Yield of Chitosan (%)
	NaOH Conc. (M)	Temp. (°C)	Time (h)	NaOH Conc. (M)	Temp. (°C)	Time (h)	
Chitin from silkworm pupa	C ₂ H ₅ OH	—	4	12,5	150	16	32,7
Chitin from cover of bees	H ₂ O ₂	75–80	1	12,5	125	1,5	16-25

Their demineralization studies were carried out using 1 – 2 N HCl for 0.3–96 h at 25 °C – 100 °C, which is stronger than the demineralization process of aquatic crustacean materials. The demineralization of shrimp waste is completed within 15 min using 0.25 M HCl at room temperature. Zhang et al. (2000) found

that the crystallinity of chitin increased and 55 % of the N-acetyl groups of silkworm chitin were removed after treatment with 2 N HCl at 100 °C. Therefore, the treatment of insect cuticle with dilute HCl is not only for removal of mineral but also for removal of the acetyl groups of insect chitin. The deproteination of insect pupa and larva was carried out by using 0.75 – 2.5 N NaOH for 2 – 42 h at 40 °C – 100 °C, which is similar to that of crustacean raw materials. The deacetylation of insect was carried out by using 10 – 12.5 M NaOH for 15 – 16 h at 110 °C – 150 °C. The degree of deacetylation of insect chitosan reaches 70 % – 95 %, this value is high then crustaceans and fungi chitosan [28].

Complex process of chitosan obtaining from chitinous cover of bees is performed for pharmacology needs, but it is expensive, so obtained drugs are also expensive and that's why are not all available. Special attention should be paid to bees' dead bodies that may be proposed as chitosan source [8].

CONCLUSIONS

Since the traditional methods of chitosan by catches of shellfish and complex selection procedures of desired product are largely limited, the needs in new sources are very acute. Such sources may be microorganisms, mushrooms and insects.

The mycelium of fungi can be considered an alternative source for the production of chitin and chitosan that might be useful for some specific practical applications. Seasonal availability does not affect on the fungal chitosan. Concurrently, the production of chitin/chitosan from fungal sources appears promising because the process can be manipulated to obtain a pure, rather uniform product with specific characteristics. In addition, the fermentative production of fungi on cheap industrial by-products and wastes is practically unlimited and, in principle, a very economic source of chitin/chitosan [28]. But the disadvantages of fungi as sources of chitosan obtaining are large amount of time spent for the cultivation and usage of a large number of equipment. These aspects make chitosan obtaining from fungi economically less profitable.

Usage of dead bees for chitosan manufacture is grounded by economical benefit. Getting of chitosan from dead bees are much cheaper and easier than by the treating of crustaceans or mushrooms. Dead bees treating require smaller number of equipment and less time. Since in Ukraine dead bees are not used in production and just ejected as waste, and annual raw material base of dead bees is about 5 thousand tons, it allows us to consider them as large-scale and perspective source of chitosan obtaining.

Thus, to meet the global demand for chitosan continuous production, it is necessary to use alternative chitosan sources, like mushrooms and dead bees.

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ПОРІВНЯЛЬНИЙ АНАЛІЗ ДЖЕРЕЛ ОТРИМАННЯ ХІТОЗАНУ

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У статті висвітлено властивості та області застосування таких біополімерів як хітин і хітозан. Розглянуто традиційні джерела отримання хітозану та проблеми, які виникають за їх використання. Запропоновано альтернативні, більш перспективні джерела отримання хітозану.

Ключові слова: *хітин, хітозан, морські організми, гриби, мікроорганізми, комахи.*

СРАВНИТЕЛЬНЫЙ АНАЛИЗ ИСТОЧНИКОВ ПОЛУЧЕНИЯ ХИТОЗАНА

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В статье освещены свойства и области применения таких биополимеров как хитин и хитозан. Рассмотрены традиционные источники получения хитозана и проблемы, возникающие при их использовании. Предложены альтернативные, более перспективные источники получения хитозана.

Ключевые слова: *хитин, хитозан, морские организмы, грибы, микроорганизмы, насекомые.*