Production of chitin from dead Hermetia Illucens

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Abstract. Searching for new ecologically and economically efficient sources of chitin is of great interest in the field of biotechnology. Nowadays, the topic is growing fast, and many scientists, researchers, primarily the representatives of the Russian Chitin Society, study and search for new sources of chitin not only from large crustaceans, molluscs, and crabs, but also from insects and small crustaceans. Domesticated and liable to breeding representatives of invertebrate animals, particularly large American Black Soldier Fly (Hermetia illucens L.) can be new and promising raw material source. The Fly is a promising object of research because it contains a chitinous external skeleton. During the studies at the Biology, Ecology and Biotechnology Department of NARFU named after M. V. Lomonosov, there were determined a sufficiently high percentage chitin yield from dead flies equal to 21.3% (1281.2 g per year per 1 m³ of a cage), almost complete absence of residual protein (C = 0.98 µg/ml) and high adsorption ability (X = 156.6 mg/g) of the extracted polysaccharide. Studied qualitative characteristics enable to consider the product as environmentally and economically cost-effective sorbent with the possibility of application in many areas of biotechnology, environmental and industrial fields of production.

1. Introduction
Chitin is one of the most naturally prevailing compounds from the polysaccharide group. It has many beneficial qualities, the main of which are protective and supporting functions of crustaceans, fungi and bacteria. Chitin provides rigidity of the cells, has healing properties, fights inflammation, improves digestion and accelerates processes of tissue recover. Potential sources of chitin are the shells of marketable shellfish, squids’ gladius (skeletal plate), cuttlefish’s sepian, biomass of micellar and higher fungi. It is known that the shells of crustaceans are rather expensive, so, despite over 15 ways to obtain chitin from them, researchers raised a problem of searching for other sources which included small crustaceans and insects. Due to the rapid reproduction domesticated and liable to breeding insects can provide a large biomass containing chitin (table 1), and according to the research by American, Russian and European scientists, insects’ chitin is 20-50 times superior to the crustaceans’ one: it doesn’t contain calcium salts and fluorine compounds [1-6]. For example, bees’ chitosan (an element derived from chitin) called apisan (translator’s note: lat. apis - bee) is a more biologically active substance than crustaceans’ chitosan. Its positive role in the regulation of all types of metabolism, as well as tonic, anti-inflammatory, adaptogenic and restorative effect has been already proved [7].
Table 1. Chitin content in insects and crustaceans.

| Material type          | Content of chitin, % |
|-----------------------|----------------------|
| Crustaceans           |                      |
| Crab shells           | 32.4                 |
| Shrimp shells         | 9.7                  |
| Shells of crayfish    | 35.0                 |
| Dried gammarus        | 26.6                 |
| Frozen gammarus       | 26.2                 |
| Antarctic krill       | 2.8–4.5              |
| Gladius of a squid    | 28.0–35.0            |
| Coleoptera insects    |                      |
| Elytra of:            |                      |
| - Colorado potato beetle | 32.2                |
| - cock chafer         | 33.9                 |
| - common stag beetle  | 40.0                 |
| - ground beetles      | 36.1                 |
| - meal worm           | 29.0                 |
| - margined water beetle | 34.5              |
| - black waterleaf     | 32.4                 |
| Cuticle of Coleoptera larvae of: |
| - meal worm           | 33.0                 |
| - cock chafer         | 33.5                 |

Along with bees, North American Black Soldier Fly (*Hermetia illucens* L.) belonging to the Soldier Family (Stratiomyidae) can also serve as a source of chitin. The species is found in countries with a warm climate, but recently it has been actively discussed that the breeding of flies is possible in regions with cold climatic conditions, and Arkhangelsk region (Russian Federation) is no exception. In the North the species can live only in the farms under artificially created conditions, owing to which it will never become invasive due to the lack of optimal parameters for the insect's growth and development in the environment of the Palaearctic region, what is very important from an environmental point of view.

Wide popularity was obtained by the insect due to highly efficient bioconversion of different organic solid wastes as well as high nutrition of the larvae applicable for feeding farm animals and aquaculture. Nowadays, the largest producer of food supplements based on larvae is the South African company "AgriProtein". It produces natural and rich in protein fodder for animals and fish. Each farm of the "AgriProtein" is capable of converting 250 tons of organic waste into protein by means of $8.5 \times 10^9$ Black Soldier Fly’s larvae, easily digestible by fish and poultry [8].

Using Black Soldier Fly as a source of chitin is a new, unique trend in the field of biotechnology on the territory of Russia and many European countries, as well as this is a promising object of improving environmental sustainability and environmental security. Similar researches were conducted only on the basis of the Natural Science University in Lublin (Poland), where the physicochemical structure of the extracted raw materials was studied (figure 1) [9].

![Figure 1. Photo of chitin from puparium (A) and imago (B) of the Black Soldier Fly.](image-url)
Research purpose is to explore the possibility of obtaining chitin from dead Black Soldier Flies and determine its percentage content and quality characteristics.

2. Methods
Breeding of Black Soldier Fly was carried out in laboratory conditions on the basis of a small innovative enterprise LLC «Biolaboratory» (Russian Federation). Experiment on the isolation of chitin was made by means of the Biology, Ecology and Biotechnology Department of Northern (Arctic) Federal University named after M.V. Lomonosov (Arkhangelsk).

A sample of dried dead flies was subjected to grinding in a planetary ball mill RETSCH PM 100 by means of large grinding balls in the grinding jar for 10 minutes at 350 rpm. With the help of analytical laboratory sieves RETSCH with mesh size of 100 µm the resulting material was sifted to separate it from impurities.

A sample of dead flies having a mass of 2.9804 g (≈ 3 g) was placed in a conical centrifuge tube with a volume of 50 ml, and washed from substrate residues with surface-active substance, and then with tap and distilled water. The washed chitin-containing fraction (mass of 2.9804 g (≈ 3 g)) was transferred to a glass conical flask (capacity of 100 cm³) having stirrer and reverse air refrigerator. Hydrochloric acid (volume of 12 cm³, concentration of 5.0 %), treatment of which was carried out at room temperature and constant stirring for 2 hours, was added to the flask. After the solution having been removed, the precipitate was washed with distilled water until neutral reaction.

The washed precipitate was mixed with aqueous solution of sodium hydroxide (volume of 12 cm³, concentration of 5.0 %) preheated to 90°C. The process of deproteinization had been lasting for 3 hours at a temperature of 100°C and constant stirring, and with no contact of the reaction medium with air. After the solution having been removed, the precipitate was washed with distilled water and 5 cm³ of ethanol. After removal of the alcohol, one added 12 cm³ of chloroform and performed fat extraction at a temperature of 50°C and stirring for 2 hours [10].

With suspension being transferred to a filter paper of the Buchner funnel, the precipitate was separated under a vacuum and dried in the lyophilizer Labconco FreeZone 2.5 L.

The yield of chitin from dead Black Soldier Flies in percentage terms was calculated according to the formula (1), %:

\[ X = \frac{m_1}{m(1-W)} \times 100 \]  

where \( m \) is the mass of the original sample, g;
\( m_1 \) is the mass of the released chitin, g;
\( W \) is the humidity of the sample, %

The moisture of the sample was determined according to the formula (2), %:

\[ W = \frac{m-m_1}{100} \]  

where \( m \) is the mass of the original sample, g;
\( m_1 \) is the mass of the released chitin, g;

Determination of chitin’s sorption ability of Black Soldier Fly was carried out by weighing 1.5 g of methylene blue and its further dissolution in distilled water in the flask having a volume of 1 litre. Obtained solution’s concentration was 1500 mg/dm³.

To build a calibration curve of dependence of absorbance on solutions’ concentration, blank solutions were prepared. 10 graduated flasks (volume of 50 cm³ each) were filled with 0.5; 1.0; 1.5; 2.0; 3.0; 4.0; 5.0; 6.0; 7.0; 8.0 ml of methylene blue joint with distilled water. The resulting solutions contained 15, 30, 45, 60, 90, 120, 150, 180, 210, 240 mg/dm³ of methylene blue per 1 dm³. The absorbance D of the blank solutions was determined by means of the photoelectrocolorimeter "UNICO – 2800" (190 – 1100 nm) with a blue filter having a wavelength of \( \lambda = 400 \) nm and the distance between the working facets of 10 mm.
A sample of chitin (0.1 g) was placed in a measuring flask with a volume of 50 cm$^3$, then a person added 25 cm$^3$ of the methylene blue’s solution, and was being stirred closed for 20 min. After that the tubes had been left for settling for 5 minutes, then the person took 5 cm$^3$ of the solution with a pipette, and determined its absorbance in a photoelectrocolorimeter. The absorbance of the solution should be within the range of 0.1 – 0.8 absorbance units. If the absorbance of the clarified solution is greater than 0.8 absorbance units, then 5 cm$^3$ of the solution are transferred to a measuring flask with a capacity of 50 cm$^3$ and diluted with distilled water up to a mark.

The corresponding value of solution’s concentration after a contact with chitin was determined for each value of the absorbance on the calibration chart along the x-axis. On the basis of the obtained with the formula (2) values adsorption capacity of chitin was calculated.

$$X = \frac{(C_1-C_2\cdot K)\cdot 0.025}{m}$$  \hspace{1cm} (3)

where $C_1$ is the concentration of the original dye solution, mg/dm$^3$; $C_2$ is the concentration of the solution after the contact with chitin, mg/dm$^3$; K is the coefficient of solution dilution taken for analysis after the contact with a polymer, K = 10; m is the mass of the chitin’s sample, g; 0.025 is the solution’s volume of methylene blue taken for clarification, dm$^3$.

The content of residual protein in the separated fly’s chitin was determined using M. Bradford’s reagent. The method consists in the interaction of the anionic form of the dye with the protein. The process solution of bovine serum albumin (BSA) was prepared by weighing 0.1 g of BSA and dissolving it in a conical centrifuge tube (volume of 15 ml) in 10 ml of distilled water. To draw the calibration curve in tubes of 2 ml one prepared BSA dilutions for a range of concentrations from 2.5 to 5 µg/ml (table 2).

| Volume of distilled water, µl | Volume and concentration of the added solution of BSA, µl | Final concentration of BSA, µg/ml |
|------------------------------|----------------------------------------------------------|----------------------------------|
| 1800                         | 200 µl, BSA solution 10 mg/ml                             | 1000                             |
| 1800                         | 200 µl, BSA solution 1 mg/ml from the tube 1              | 100                              |
| 1900                         | 100 µl, BSA solution 1 mg/ml from the tube 1              | 50                               |
| 1950                         | 50 µl, BSA solution 1 mg/ml from the tube 1               | 25                               |
| 1800                         | 200 µl, BSA solution 100 µg/ml from the tube 2            | 10                               |
| 1900                         | 100 µl, BSA solution 100 µg/ml from the tube 2            | 5                                |
| 1950                         | 50 µl, BSA solution 100 µg/ml from the tube 2             | 2.5                              |

Dilutions of BSA were mixed with Bradford’s reagent in the test tubes with a volume of 1.5 ml. In each tube there were added 800 µl of standard BSA dilutions (table 2) and 2 mg of the chitin’s sample with unknown concentration which was preliminarily diluted in water to form a suspension. To make a checkout sample, the tube №0 was filled with 800 µl of distilled water, so the measurements in photocolorimeter were made relative to this tube. 400 µl of Bradford’s reagent was added to each tube, then their contents were mixed with the help of a centrifuge – vortex Microspin FV-2400. During the analysis we observed the reagent colour change: after having added it to the protein solution, the dye combined with them, and the reagent was dyed blue. The tube with the chitin’s sample was centrifuged in Eppendorf MiniSpin, 220 VAC. The tubes were incubated for 5 minutes at room temperature. We set spectrophotometer "UNICO-2800" (190 – 1100 nm) at a wavelength of 595 nm, and measured absorption value of the solution from the test tube №0 - this very value was set as reference point when drawing the calibration curve. After that we made measurements of standard dilutions (starting with the lowest concentration) and chitin’s sample (diluted suspension). Using obtained values in the measurement of standard dilutions, it was a standard curve built, and the latter was used to calculate the protein concentration in the sample.
3. Research results

Analysis of the chitin content carried out at the Biology, Ecology and Biotechnology Department of NARFU proved the possibility of using dead Black Soldier Flies as a source of the studied component. According to the research results, the yield of chitin was 21.3 %. The finished product is of dark grey colour with a flake size of not greater than 0.5 mm. It has a specific odour due to washing with chloroform, which is unacceptable for using in food industry and manufacturing drugs for medical and veterinary purposes.

At the stage of demineralization, the mineral part of the polysaccharide was removed. Chitin was subjected to alkaline and enzymatic extraction of protein and was separated from the fat using chloroform. As a result, the weight of the finished product amounted to 0.6229 g.

Moisture of the sample was as follows:

\[ W = \frac{(2.9804 - 0.6229)}{100} = 0.0235\% \]

The yield of chitin from dead Black Soldier Flies in percentage terms was calculated according to the formula (1), %:

\[ X = \frac{0.6229}{2.9804(1 - 0.0235)} \cdot 100 = 21.3\% \]

The absorbance of the clarified solution was \( D = 2.459 \) absorbance units; as the value exceeds 0.8 absorbance units, 5 cm\(^3\) of solution were transferred into a graduated flask and diluted with distilled water. The final value is \( D = 0.241 \) absorbance units.

Using calibration curve, we determined the residual concentration of methylene blue in the clarified solution: \( C = 87 \text{ mg/dm}^3 \) (figure 2).

![Figure 2. Determination of the residual concentration of methylene blue in a clarified solution.](image)

Adsorption activity of chitin was as follows:

\[ X = \frac{(1500 - 87 \cdot 10 \cdot 0.025)}{0.0998} = 156.6 \text{ mg/g} \]

Adsorption activity is a specific indicator of the sorbents quality and is used to characterize their absorption capacity. In comparison with crustaceans’ chitin (60-65 mg/g), chitin of Black Soldier Fly has high adsorption capacity that is 156.6 mg/g, which indicates the ability to bind a huge range of substances of organic and inorganic nature, thereby expanding field-of-use of this biopolymer.

On the basis of the analysis for determining the concentration of protein in the sample of the isolated Black Soldier Fly’s chitin by Bradford’s method it was established that the absorbance of the solution relative to the sample with distilled water was \( D = 0.005 \) absorbance units (figure 3). On the basis of the
calibration curve's equation the protein concentration in chitin’s suspension amounted to 0.98 µg/ml; obtained value can be regarded as an error, so we can conclude the protein is absent.

![Graph](image)

**Figure 3.** Calibration graph of absorbance's dependence on the concentration of the standard BSA dilutions.

Obtaining chitin from Black Soldier Fly on the basis of "Nordtest" is a subsidiary activity to the main product being produced at the enterprise. The main goal of the company is production of flies’ larvae’s and prepupa’s biomass as high-protein food additive for cattle and aquaculture. Cages with a volume of 0.8 m$^3$ are used for the maintenance of imago. Each cage contains about 4000 flies. When calculating a number of imago per 1 m$^3$, it equals to about 5000 adults. According to researches, the average weight of 1 dried fly is 0.0341 g., hence, the mass of the total number of flies in a cage amounts to 5000*0.0341 = 170.5 g / 1 m$^3$, while a part of chitin (21.3%) equals to 36.32 g. A fly goes through five stages during the life cycle: egg, larva, prepupa, pupa and imago. Minimum number of laying cycles of species into cages at intensive species’ breeding technology during the production for 1 year is 35 units, while the amount of chitin per 1 m$^3$ of a cage equals to 36.32*35 = 1281.2 g/year. The stated above yield of chitin from about 5000 flies is economically advantageous indicator which shows efficiency and economic conditionality of additional raw materials, with the possibility of its use in many areas of biotechnology, ecology and industry.

4. Discussion
Researching established that the yield of chitin from adult specimen (dead specimen) of Black Soldier Fly is rather high and amounts to 21.3 %. The number of extracted from the fly raw materials exceeds that of some crustaceans 2.1...7.1 times (table 1) in comparison with the data from information sources. However, in comparison with the insects the fly *Hermetia illucens* is inferior to them in most cases so it is defined to be not the most promising chitin-containing object of study.

High adsorption capacity (X = 156.6 mg/g) of the isolated fly’s chitin and almost complete absence of protein (C = 0.98 µg/ml) enable to consider this product as a cost-effective sorbent being able to bind enormous range of organic and inorganic substances. Economic efficiency of the raw materials is represented by a chitin's amount obtained per 1 m$^3$ of an imago's cage per year - 1281 g/year, and by less number of chemical reactions carried out to determine the content and to remove residual protein.

Ecological orientation of the studied fly is connected with larvae’s ability of organic wastes bioutilization, what increases environmental safety and enables to solve a number of biological problems in the Russian North.

Revealed chitin's qualitative characteristics along with ecological and economical orientation of the fly define Black Soldier Fly as an object of great scientific and practical interest, and provide an
opportunity to apply it in many areas of industry and biotechnology in regions with cold climatic conditions.

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