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VALORIZATION OF MUSSEL WASTE FROM THE ROMANIAN BLACK SEA COAST

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ABSTRACT

In this study the shells and byssus of *Mytilus galloprovincialis*, the mussels from the Romanian Black Sea coast were studied through biotechnological techniques, to determine if they can be highly economically valorized. The shells were processed in order to obtain calcium carbonate ($CaCO_3$) and subsequently calcium chloride ($CaCl_2$), natural compounds with multiple practical applications in the pharmaceutical industry, medicine, agriculture, animal husbandry, environment, etc. Calcium carbonate was obtained from mussel shells with a yield of 95.31%, and calcium chloride was obtained with a yield of 73.80% from $CaCO_3$, percentages that support the efficiency of the biotechnological processes for obtaining these products.

The byssus collected from *M. galloprovincialis* was processed using a pepsin-aided extraction method, which allowed obtaining collagen with a yield of 27,56%. Mussel byssus is a waste and may provide an alternative to other types of existing collagen from animal sources with different applications in pharmaceutical industry, tissue engineering, medical field, food industry, cosmetics.

Keywords: *Mytilus galloprovincialis* waste, calcium carbonate, calcium chloride, collagen, Black Sea coast

AIMS AND BACKGROUND

Edible parts of mussels are small and thus large amounts of shells and byssus as waste material are produced from their processing in the seafood industry (Nyein *et al.*, 2019). The disposal of mussels' waste in environment after their processing may lead to different problems generated by quantities, storage places, natural degradation (Monneron-Gyurits *et al.*, 2018).

The shells and byssus of *M. galloprovincialis* from the Romanian Black Sea coast can be superior economically valorized through biotechnological techniques.

Calcium carbonate, calcium chloride and collagen extracted from *M. galloprovincialis*, a natural resource, represent autochthonous products which can be used in different domains and to solve environmental problems.

Marine organisms are a major source of food which are gaining a lot of interest from the scientific community for their nutritional value, and potential role in the functional food and nutraceuticals industry. The health-promoting effects of many products isolated from marine organisms have been established after successful clinical trials (Coulson *et al*., 2012; Coulson *et al*., 2013; Kean *et al*., 2013; Stebbings *et al*., 2017).

M. galloprovincialis is a source of omega 3 polyunsaturated fatty acids and fat-soluble vitamins (A, D and E). Many molluscan species find use in traditional medication in different parts of the world (Chakraborty *et al*., 2009). Molluscan shells (Latire *et al*., 2009), soft tissue, basal parts, mucilage, and even the entire organisms (Kehinde *et al*., 2015) have been traditionally used as medication for curing gastrointestinal ailments, cancer, inflammations, dotage, and some other diseases.

Calcium is an essential nutrient for many functions in human health. It is the most abundant mineral in the body, 99% found in teeth and bones and only 1% in serum. Research has shown that adequate calcium consumption can help reduce the risk of fractures, osteoporosis and diabetes in some populations. The dietary requirements of calcium and other collaborative nutrients vary slightly across the globe (Beto, 2015).

There are several types of calcium supplements in pharmacies, each with its own characteristics and indications such as: calcium carbonate, lactic calcium, calcium citrate, calcium gluconate, calcium chloride. These synthetic pharmaceutical products can be supplemented or replaced by products of marine origin (forms of calcium extracted from shells) which have the advantage of being natural, easy to obtain, indigenous and can solve the problem of waste (shells) resulting from processing for alimentary and which most often end up in the environment.

Mussel shells are potential candidates for the extraction and use of calcium with various applications, especially medical and pharmaceutical. Calcium is a micronutrient involved in multiple biological functions, including nerve impulse transmission, muscle contraction, intracellular signaling, blood coagulation, etc.

Calcium chloride is used to combat sleet, ice and snow on roads, in the oil extraction, chemical and paper industries, in cold technology, in construction, non-ferrous metallurgy (https://www.arcadiamedical.ro/articol/de-ce-apare-lipsa-de-calciu-si-cum-o-tratam/; https://www.carbonatdecalciu.ro/(*in Romanian*).

Collagen is the most essential extracellular protein in an animal's body, involved in the mechanical protection of tissues and organs. It has a long history as a natural material and has been used in different biomedical fields, such as tissue engineering and drug delivery systems (Liu *et al*., 2018). Due to their increased demand and growing application in dental surgeries, tissue engineering, bone grafting, food and pharmaceutical industries, extraction of collagen has developed (Avila Rodriguez *et al*., 2018; Mofieed *et al*., 2020).

Collagens have been elected as one of the key biological materials in biomedical applications due to their high biocompatibility (Silva *et al*., 2014).

The outbreaks of bovine spongiform encephalopathy and foot-and mouth disease crisis, restrictions on collagen trade have been established and alternative safe sources of collagen are needed. Another problem of bovine collagen application is the risk of autoimmune and allergenic reactions (Zhao and Chi, 2009).

Byssal threads of mussels are composed of over 50% collagen (Harrington and Waite, 2008), although differences in concentration had been shown between species (Lucas *et al*., 2002). In addition, it has been highlighted that *‘‘byssal collagens have no match with any known collagen sequence’’* (Qin and Waite, 1995).

Mussel processing results in the generation of a large waste biomass (i.e., byssus and shells) which is generally discarded. If substantial amounts of collagen could be obtained from this waste (byssus), it would provide alternatives to mammalian collagen in food, pharmaceutical and biomedical materials using the same approaches for collagen extracted from marine invertebrate sources.

EXPERIMENTAL

Mussels (*M. galloprovincialis*) harvested by divers from the southern part of the Romanian Black Sea coast (Vama Veche area), in August 2020. The biological material was analyzed in the Marine Biochemistry Laboratory within National Institute for Marine Research and Development (NIMRD) "Grigore Antipa" Constanta. The next step was washing with running water and the meat, shell and byssus were separated. Selected mussel shells were subjected to bleaching (depigmentation) step by immersing in acetone 100% for 20 minutes and allowed to dry naturally for 4 hours (Fig.1).

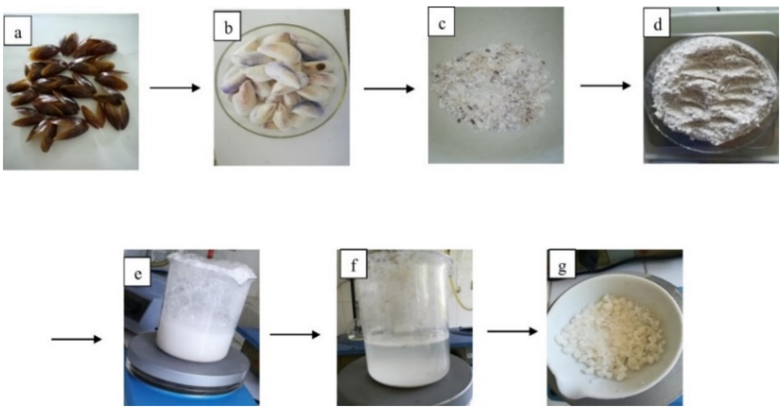

Fig. 1. Flow chart describing the extraction method of calcium carbonate and calcium chloride: a) Mussel shells; b) Depigmentation and bleaching with acetone and sodium hypochlorite; c) Grinding the shells, obtaining calcium carbonate; d) Grinding, obtaining homogeneous calcium carbonate powder; e) Treatment with hydrochloric acid; f) Obtaining the aqueous solution of calcium chloride; g) Crystallized calcium chloride.

The bleaching step was performed by treating the dried shells with sodium hypochlorite solution 35% for 5 hours. The depigmented shells were rinsed with tap water, followed by washing with ultrapure water and kept in oven for 4 h at 110°C. $CaCO_3$ powder was prepared by crushing and grinding the dry shells by mortar. The obtained powder was dried in oven for 2 h at 110 °C to ensure complete drying. Finally, the product was sieved into <1 mm fractions and used in the subsequent step for $CaCl_2$ preparation (aq) by treatment with 1M HCl following a method described by Elgarahy *et al*., 2020 (Fig. 1).

Collagen from fish wastes (skin, bones and scales) is the most studied source for scaffold design; but its low denaturation temperature and variable composition limits its application in this field (Subhan *et al*., 2015). Hence, invertebrate marine collagen sources are now under investigation. Scaffolds from jellyfish and marine sponges' collagen have been developed, obtaining materials with high porosity, interconnected porous structure, high cell viability and no cytotoxicity (Hoyer *et al*., 2014). These promising results lead to further research in the area.

The byssus harvested from mussels was used for collagen extraction using the enzymatic pepsin method described by Rodriguez *et al*., 2017 (Fig. 2).

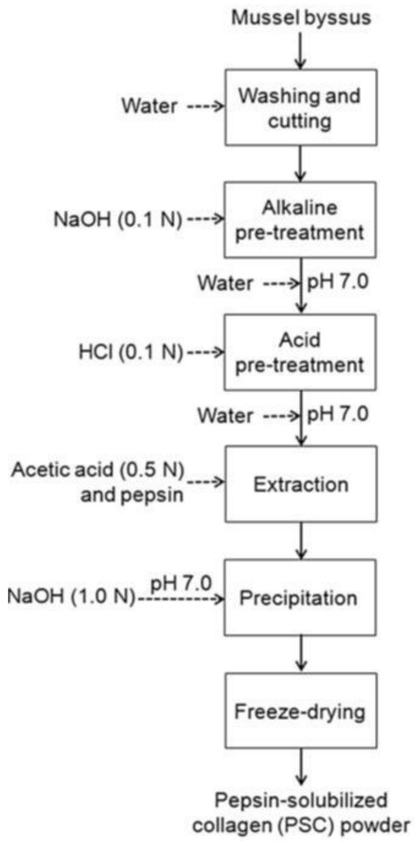

Fig. 2. Flow chart describing the extraction method of collagen from mussel byssus by using the enzymatic extraction procedure

The byssus was washed and minced by cutting, dried, then subjected to a pre-alkaline treatment with 0.1N NaOH to solubilize non-collagenous proteins, followed by a pre-acid treatment with 0.1N HCl to collagen solubilization and material demineralization. Between, the byssus was rinsed with distilled water to reach pH 7.

For collagen extraction, pepsin solution 800–2500 units/g protein in 0.5N acetic acid, pH adjusted to 1.5 with 5N HCl, used in a pepsin/byssus ratio of 4:50 w/w for 24 hours at 25^{o}C. After the enzymatic treatment, the solution was filtered and put into test tubes to inactivate the enzyme by applying a thermal treatment at 98^{o}C for 1 min. The solution was adjusted at pH 4.0 with 5N NaOH solution and the byssus was put back into the acetic acid solution. Samples were heated at 80^{o}C for 24 h for collagen extraction, then, the dispersion was filtered under vacuum. The filtrate was recovered and precipitation was carried out by adjusting the pH to 7.0 with 1N NaOH and the dispersion was centrifuged at 5000 rpm. The supernatant was discarded, the pellet was freeze-dried and is considered pepsin solubilized collagen powder (PSC).

The huge number of marine species having very different intrinsic characteristics has aroused the scientific and commercial interest in optimizing the extracting conditions, as well as characterizing the yields, physicochemical and functional properties of marine collagen (Silva *et al*., 2014).

RESULTS AND DISCUSSION

Mussels (*M. galloprovincialis*) from the Romanian coast of the Black Sea are sedentary organisms, and because they cluster in relatively stable populations, this suggests the accessibility of the source. In general, the production of mussels is marketed for food purposes, but it is a capitalization conditioned by their size. Although there are no specialized concerns at an industrial level in the field of mussel exploitation in the Romanian coast area, nevertheless the presence on the market of this natural food resource, justifies and supports the biotechnological valorization approach (Crâsmaru *et al*., 2004).

In this study, *M. galloprovincialis* shells were processed in order to obtain calcium carbonate $CaCO_3$ and subsequently calcium chloride $CaCl_2$, natural compounds with multiple practical applications.

The stages of processing the shells and obtaining the mentioned products are briefly shown in Fig.1. Calcium carbonate $CaCO_3$ was obtained in 95.31% yield from the shells (Yield% = [$CaCO_3$ (g)/ shell powder (g)] x 100%), and calcium chloride $CaCl_2$ was obtained in 73.80% yield from $CaCO_3$ shells (Yield% = [$CaCl_2$ (g)/ $CaCO_3$ (g)] x 100%).

Mollusc shells have drawn many researchers' interests in materials science, nanotechnologies and biology due to their unique biological and physical properties (Istin, 1975). They are a cheap source of calcium carbonate, calcium hydroxide, as well as a nutritional supplement in poultry and cattle feed, as an absorbent for acid gases emitted by power plants and incinerators. Another use of shells is in household water treatments.

Calcium carbonate and calcium chloride are quite used in the economy, representing a safe source of marine origin (mussel shells) that cover the current market requirements (https://www.arcadiamedical.ro/articol/de-ce-apare-lipsa-de-calciu-si-cum-o-tratam/; https://www.carbonatdecalciu.ro/).

Mussels (*Mytilus sp.*) are found in different habitats and adhere to surfaces by byssus (Fig. 3). Byssus is a structure composed of individual threads representing a composite reinforced with collagen fibers, that play an important role in withstanding wave forces (Harrington and Waite, 2008).

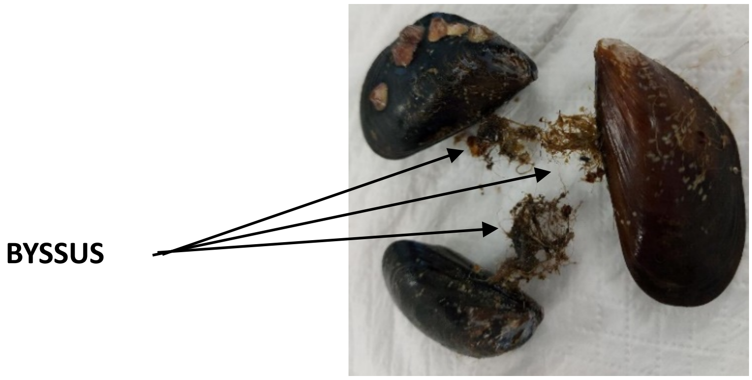

Fig. 3. *Mytilus galloprovincialis* mussel byssus from the Black Sea (original photo)

The generated waste by mussels processing (byssus and shells) is a material that usually ends up in landfills. The byssus represents a source of collagen that can be extracted and utilized in different directions.

Pepsin solubilized collagen powder (PSC) from mussel byssus was obtained with an extraction yield of 27.56% (Yield% = [PSC collagen (g)/ dried byssus (g)] x 100%). The obtained collagen has not been studied from the point of view of stability: pH effect on solubility, denaturation temperature. These aspects will be further investigated.

Marine collagen sources with potential application in tissue engineering are novel candidates to be used for biomedical applications (Silva *et al.*, 2014; Subhan *et al.*, 2015). It is known that marine collagen has many advantages over terrestrial animals, such as: (1) high collagen content; (2) no risk of diseases such as BSE (bovine spongiform encephalopathy), TSE (transmissible spongiform encephalopathy, FMD (foot-and-mouth disease); (3) no religious and ethical conflicts; (4) biological toxins and contaminants are present in smaller quantities; (5) less immunogenic; (6) low inflammatory response and (7) metabolically adequate (Mofieed *et al.*, 2020).

Marine collagen extraction opens an opportunity for further research to establish the functional activities of mussel byssus collagen, as a new potential source. All these results could be used as a basis for crafting strategies to assist the reutilization of waste and by-products from mussel processing plants (Rodriguez *et al*., 2017).

The Black Sea offers enough resources that can be valorized and at the same time some of the waste problems from processing plants can be solved (in the present case of mussels).

CONCLUSIONS

✓ Significant quantities of shells and byssus resulting from the processing of mussels' meat represent unutilized waste, but extremely valuable from the point of view of the biochemical compounds contained. Mussel shells are a source of organic material, accessible for marine biotechnology in order to obtain calcium carbonate and calcium chloride.

✓ Calcium carbonate $CaCO_3$ resulted from mussel shells with a yield of 95.31%, and calcium chloride $CaCl_2$ with a yield of 73.80% from $CaCO_3$ sustain the efficiency of biotechnological process.

✓ Marine collagen extracted from the Black Sea mussel byssus (yield 27.56%) is an alternative source of collagen derived from terrestrial animals which is controversial due to the problems related to diseases, religion, etc.

✓ This research is an attempt to evaluate the valorization possibilities of waste resulting from *M. galloprovinciallis* processed in the food industry. At the same time, could be a solution to the environmental problems generated by waste discarded.

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