



(RESEARCH ARTICLE)



Recovery of glucosamine and optimization of chitin extraction with acid and alkali process from shells of sea mussel *Mytilus galloprovincialis* in Greece

Kostas Katsoulis *

Department of Animal Husbandry and Nutrition, University of Thessaly, Faculty of Veterinary Science, Trikalon 224, Karditsa, 4313-1, Greece.

GSC Biological and Pharmaceutical Sciences, 2021, 16(02), 166–171

Publication history: Received on 16 July 2021; revised on 18 August 2021; accepted on 20 August 2021

Article DOI: <https://doi.org/10.30574/gscbps.2021.16.2.0245>

Abstract

Mussel wastes include byproducts such as seed, barnacles fouling, broken shells, byssus threads, which are associated with serious environmental impacts on aquatic ecosystems. The objective of reducing wastes and to avoid environmental problems can be achieved by establishing alternative methods to valorize mussel wastes converting them into high value-added products.

The present study aims to obtain by chemical methods the extraction of chitin and glucosamine from shells of *Mytilus galloprovincialis*. Chitin, which chemically is a linear polysaccharide of β (1→4) linked *N*-acetylglucosamine monomers, possesses multifunctional properties and is suitable for various applications mainly in pharmaceutical, biomedical food and packaging fields. Glucosamine (C₆H₁₃NO₅) is an amino sugar (2-amino-2-deoxy- β -D-glucopyranose) and is a part of the structure of two polysaccharides, chitosan and chitin. Due to its high aqueous solubility and physical stability is used as a potential solid-dispersion carrier to improve the biopharmaceutical properties of drugs.

The influence of temperature, solid/liquid ratio (g/ml), molarity of solutions and agitation on chitin production and acid hydrolysis of chitin was studied to achieve optimum output of chitin and glucosamine.

Our results demonstrates that the production of glucosamine and chitin is greatly influenced by the experimental conditions. The best yield for glucosamine was obtained at a solid/liquid ratio of 1:15 at 60°C for 1 hour.

Keywords: *Mytilus galloprovincialis*; Chitin; Glucosamine; Mussel shell; Extraction

1. Introduction

The Mediterranean mussel *Mytilus galloprovincialis* is the cultivated species in Greece and more generally in the Mediterranean area. Longline culture is the most recent development for mussel culture in Greece. A horizontal longline rope is suspended by a series of anchored floats, and ropes or socks of mussels are hung from this rope. The mussel *Mytilus galloprovincialis* displays great tolerance to environmental variability. This species grows in waters with a salinity of 32-37‰ while it survives without problems and in sea waters which salinity ranges from 22-42‰. In addition to salinity, the temperature also plays an important role to the growth of the mussel. The temperature ranges from 10 and 26°C with an excellent of 15-19°C and the pH value ranges from 7.0 to 8.3. Mussels grows to market size when they reach 5 - 7 cm long, usually at the end of the spring, 15 -18 months after the harvesting of the seed from the spat collectors [1,2]. Interactions between mussel culture and the environment such as nutrient cycling, biodeposition and benthic effects, have been studied thoroughly [1,3,4,5]. The increase in sedimentation below mussel culture is due

* Corresponding author: Kostas Katsoulis

Department of Animal Husbandry and Nutrition, University of Thessaly, Faculty of Veterinary Science, Trikalon 224, Karditsa, 4313-1, Greece.

mainly to the excretion of faeces and pseudofaeces by mussels or to dead shells [1]. The mussel shell is a complex organic/inorganic system comprising two calcium carbonate polymorphs: calcite (prismatic layer) and aragonite (nacreous layer) coated by an outer organic layer, the periostracum [6,7]. Mussel shells have not been used optimally so that they are discarded in the environment. The objective of the present study was to assess the efficiency of different extraction methods on recovery of bioactive polymers of mussel shells, such as chitin and glucosamine, adding value to a valueless waste product.

2. Material and methods

The mussel culture was selected in a specific marine area of the Prefecture of Pieria (Makrygialos - latitude: 40° 24' 51, 8688" N, 22° 36' 39, 4848" E). The mussel culture was of the "long line" (floating) type. The distance of the aquaculture from the coast was 700 m and its depth water was 15 meters. The experimental harvest of mussels was carried out in September 2020. A total number of 126 specimens were collected. Firstly, the shells were removed from the animal and secondly the specimens were packed in polyethylene bags, placed on ice, transported to the laboratory and were stored in a freezer at -20°C until further use.

2.1. Reagents

All the chemicals and solvents used (sodium hydroxide, hydrochloric acid, acetic acid, potassium hydroxide, anhydrous sodium sulfate, petroleum ether, ethanol) were purchased from Sigma-Aldrich, were of analytical grade and used without purification. A commercial N-acetyl-D-glucosamine reagent was chosen from Alfa Aesar Chemicals. All solutions were freshly prepared in distilled water.

2.2. Methods

Drying of samples was obtained by heating in a drying oven (model R. Espinar, S.L.) at 90°C until constant weight was obtained between two sequential measurements [8,9]. Samples grinded in a laboratory mill (System POLYMIX® PX-MFC 90 D) into smaller particles using sieve with 1 mm wide openings. pH measurements were made using a digital laboratory pH meter (model Mettler Toledo MP 220) which was calibrated using certified pH= 4.0 and pH= 7.0 buffer solutions, according to the official method [8]. The ether extract (EE) was determined using method of Soxhlet. Approximately 3 g of solid sample were mixed with anhydrous sodium sulfate, placed in an extraction thimble and were extracted using an appropriate solvent in the Soxhlet extractor (diethyl ether). Ash contents were determined using dry ashing method. The samples (2 g) were ashed for about 8h until a grey ash residue had been obtained using a furnace (model P. Selecta, 3000 W) where temperature had been gradually increased from room temperature to 450°C in 1 hour [8,9].

2.3. Extraction of chitin and glucosamine recovery by chemical method

A total of 10 - 18 g dry samples of mussel shell waste were treated with 1.3M KOH/ 1M NaOH at solid to solvent ratio 1:5 and 1:7 (w/v), with constant stirring at 400 rpm or not, for 48 hours at room temperature, with pH ranged from 11-13 (removing of proteins). After that, the solution was filtered and the samples were washed with distilled water to neutrality in running tap water. Water from the samples was removed before performing the demineralization process.

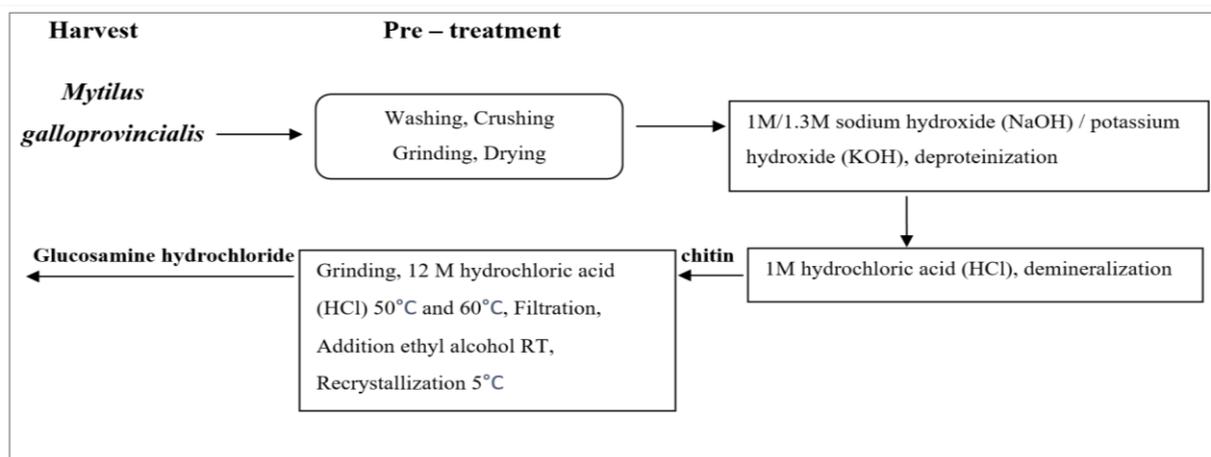


Figure 1 Flow diagram of chitin and glucosamine extraction from shells of *Mytilus galloprovincialis*

Samples from deproteinization process were treated with 1.0 M HCl in the ratio 1:8 and 1:10 (w/v), with constant stirring at 400 rpm for 48 hours with pH value ranged pH 1.0-2.5 at room temperature (RT). The solution was filtered and the samples were washed with distilled water to remove acid and calcium chloride. The samples were then dried for 3 hours using an oven at 80°C until constant weight was obtained (chitin). To obtain glucosamine, chitin was grinded to fine particles (2 mm), hydrolyzed with 12M HCl at 50 and 60°C, then was filtrated by gravity to remove the solids and finally to recover glucosamine addition of ethyl alcohol (95%) 5°C was performed. The mixture was cooled for 3 weeks to crystallize and finally the solid crystals were washed with ethanol and dried in an oven at 50°C for 8 hours.

2.4. Chitin yield and glucosamine recovery

The percentage of the yield of chitin was calculated by dividing the weight of extracted chitin to initial dry shrimp shell weight. Yield of chitin was calculated as follows:

$$\text{Yield of chitin (\%)} = (\text{extracted chitin, g}) / \text{dry mussel shells, g}) * 100$$

The percentage of the recovery of glucosamine was calculated by dividing the weight of extracted glucosamine to initial dry chitin weight. Recovery of glucosamine was calculated as follows:

$$\text{Recovery of glucosamine (\%)} = (\text{extracted glucosamine, g}) / \text{dry chitin, g}) * 100$$

3. Results

Parameters and details of experiments are demonstrated in Table 1. Some differences can be observed attributed to the changes of molarity of solutions (NaOH, KOH, HCl) for deproteinization and demineralization and to the ratio of solid to solvent. As a result, the percentage of yield differs in all experiments. The percentage of yield of chitin was presented in Table 2. The percentage of yield of chitin varied from 57.7% to 62.72%, the higher value of yield of chitin after demineralization was observed in exp III (62.72%) while the lowest value was observed in exp I (57.7%). Experimental details and recovery % of glucosamine was presented in Table 3. The percentage of yield of glucosamine after acid hydrolysis varied from 9.5% to 11%. The higher values of recovery were observed in exp V (11%) while the lowest values were observed in exp VI (9.5%). Functional and physicochemical properties of chitin that have been studied in this work have shown a variety of characteristics as it is demonstrated in Table 4. The color of chitin was whitish slightly gray, it was insoluble in water while was almost completely dissolved in acetic acid (93%) and values of ether extract and ash content were below of 1%.

Table 1 Experimental details

Parameters	Experiment			
	I	II	III	IV
Molarity of solution for deproteination (Dp)	1.3N KOH	1.0N NaOH	1.3N KOH	1.0N NaOH
Solid to solvent ratio	1:7	1:5	1:5	1:7
Yield % after deproteination	96.39	95.66	96.00	95.00
Solution Molarity for demineralization (Dm)	1.0 N	1.0 N	1.0 N	1.0 N
Solid to solvent ratio	1:8	1:8	1:10	1:10
Yield % after demineralization	57.7	59.31	62.72	60.40
Rpm	without	without	400	400
Time (d) / temperature	2 d/ room temp			

Table 2 Yield % of chitin

Experiment	Mussel shell (g)	Chitin (g)	Chitin yield (%)
I	10.00	5.77	57.70
II	13.04	7.73	59.31
III	18.60	11.67	62.72
IV	15.79	9.54	60.40

Table 3 Experimental details and recovery % of glucosamine

Experiment	Chitin (g)	HCl (ml)	ratio	Molarity of HCl (M)	rpm	Time (h) / Temperature (°C)	Glucosamine recovery (%)
V	1	15	1/15	12 M	400	1/60	11
VI	2	20	1/10	12 M	400	2/50	9.5
VII	1	20	1/20	12M	400	24/room temperature	9.85

Table 4 Physicochemical and functional properties of chitin

Parameters	Value
pH	8.6
ether extract	<1%
Ash	< 1%
solubility in water	Insoluble
solubility in acetic acid	Almost completely dissolved
Colour	Whitish slightly gray

4. Discussion

This study aimed at the utilization of mussel shell waste for production of chitin and glucosamine. These biopolymers have a wide range of applications in areas such as agriculture, pharmaceuticals, cosmetics, food and beverages and wastewater technology [10,11]. Chitin is the second most abundant no toxic biopolymer after cellulose in nature, biodegradable and mainly shows antimicrobial action against a lot of gut pathogens. Glucosamine, in the form of glucosamine hydrochloride or sulphate, is widely used as a dietary supplement and has been promoted to stimulate synovial fluid synthesis, inhibit degradation, and improve healing of articular cartilage [6,10,12].

Greece produces thousands of tons of mussels annually in longline mussel cultures (15.000 – 20.000 tons for the last five years) and wastes are available in large quantities. However, these wastes are not utilized and discarded in the aquatic environment. The use of mussel shells could be a low-cost alternative method to obtain value added products, such as chitin and glucosamine-HCl.

Our data reveal a variety of the percentage yield of chitin and glucosamine. These values were also correlated with other studies and note the influence of temperature, solid/liquid ratio (g/ml), molarity of solutions (NaOH, KOH, HCl) and agitation on chitin production and acid hydrolysis of chitin [13,14,15,16,17].

In our experiment results of 1.3 N KOH for deproteination, 1.0 N solution of HCl for demineralization at a solid to liquid ratio of 1:10 with agitation for 48 hours clearly demonstrate a significant yield of chitin. As widely reported by various studies worldwide [18,19,20,21,22,23,24] functional and physicochemical properties of chitin indicate a good quality product with valuable properties. Solubility is an important parameter which determines the quality of chitin. Chitin shows almost high solubility in acetic acid while is insoluble in water referring to a good quality product. The lower values of ash and ether extract content because of the completion of demineralization and deproteinization prove the purity of the sample. Additionally, the whitish slightly gray color enhances the above conclusions and indicates the removal of more proteins and inorganic materials from mussel waste.

The production of glucosamine is greatly influenced by the experimental conditions [12,18,25,26]. It was observed that the recrystallization process was slow at room temperature, so ethyl alcohol was added to increase the crystallization rate. The low temperature (5°C) and the use of this solvent strengthened the formation of glucosamine crystals. Moreover, chitin was dissolved within the first 25 minutes giving a yellow – brown color in the solution because of a Maillard reaction. The best yield of glucosamine (11%) was obtained at a solid to liquid ratio of 1/15, using for acid hydrolysis a concentrated solution of HCl 12M for 1 hour at 60°C with agitation (400 rpm). When the chitin was hydrolyzed at 60°C for 1 hour the yield was 11% while the yield decreased for a longer period of hydrolysis due probably to the decomposition of glucosamine hydrochloride to other compounds. The increase in temperature (60°C instead of 50°C) combined with the use of agitation dissolved the chitin faster and the yield of hydrolysis in this experiment was more effective. However, to the best of our knowledge, no studies are available in Greece on extraction of chitin and glucosamine from mussel *Mytilus galloprovincialis*. Concerning the parameters of the experiments and the percentage yield of chitin and glucosamine it is believed that the extraction process could be improved on recovery of bioactive polymers of mussel wastes.

5. Conclusion

Mussel culture in Greece produces large amounts of wastes that are discarded in the aquatic environment. Alternative processes and methods in seafood industries may help to enhance and transform mussel wastes in valuable biopolymers, such as chitin and glucosamine, covering a wide range of applications. Mussel shell processing would successfully minimize the environmental pollution; however, it is necessary to perform a greater number of experiments and the reaction conditions should be optimized to improve the glucosamine yield.

Compliance with ethical standards

Acknowledgments

Special thanks and sincere appreciation to Assistant Professor P. Pantazis, Director of the Laboratory of Department of Animal Husbandry and Nutrition at the Thessaly University for his assistance, use of laboratory space and equipment.

Disclosure of conflict of interest

The authors declare that they have no conflicts of interest.

References

- [1] Katsoulis K, Rovoli M. Potential Impacts of Mediterranean *Mytilus galloprovincialis* Mussel Farming in a Specific Area of Aegean Sea. *Journal of Veterinary Science and Medicine*. 2019; 7(1): 1- 10.
- [2] Katsoulis K. Filtration rate, oxygen consumption, phosphorous and nitrogenous excretion of *Mytilus galloprovincialis* and effect in aqueous environment, *Fresenius Environmental Bulletin*. 2017; 26(12A): 7825 – 7831.
- [3] Jansen HM, Verdegem MC, Strand O, Smaal AC. Seasonal variation in mineralization rates (C-N-P-Si) of mussel *Mytilus edulis* biodeposits. *Mar Biol*. 2012; 159(7):1567-1580.
- [4] Grant J, Hatcher A, Scott DB, Pocklington P, Schafer CT, Winters GV. A Multidisciplinary Approach to Evaluating Impacts of Shellfish Aquaculture on Benthic Communities. *Estuaries*. 1995; 18: 124-144.
- [5] Nizzoli D, Welsh D, Fano E, Viaroli P. Impact of clam and mussel farming on benthic metabolism and nitrogen cycling, with emphasis on nitrate reduction pathways. *Marine Ecology Progress Series*. 2006; 315.

- [6] Naik AS, Hayes M. Bioprocessing of mussel by-products for value added ingredients. *Trends in Food Science & Technology*. 2019; 92: 111-121.
- [7] Smithmaitrie R, Jaafar SNT, Salaenoi J. Shell structure investigation and utilization of bio-extract fermented from green mussel shell. *Agr Nat Resour*. 2020; 54: 415–422.
- [8] AOAC. *Official Methods of Analysis*, 15th ed. Association of Official Analytical. 1990.
- [9] AOAC *Official Method of Analysis*. 16th Edition, Association of Official Analytical. 2002.
- [10] Santos VP, Marques NSS, Maia PCSV, Lima MAB, Franco LO, Campos-Takaki GM. Seafood Waste as Attractive Source of Chitin and Chitosan Production and Their Applications. *Int J Mol Sci*. 2020; 21(12): 4290.
- [11] Hamed I, Özogul F, Regenstein JM. Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review. *Trends in Food Science & Technology*. 2016; 48: 40–50.
- [12] Benavente M, Arias S, Moreno L, Martinez J. Production of Glucosamine Hydrochloride from Crustacean Shell. *Journal of Pharmacy and Pharmacology*. 2015; 3(1): 20-26.
- [13] Abdulkarim A, Isa MT, Abdulsalam S, Muhammad AJ, Ameh AO. Extraction and characterization of chitin and chitosan from mussel shell. *Civil and Environmental Research*. 2013; 3(2): 108-114.
- [14] Nouri M, Khodaiyan F, Razavib SH, Mousavi MA. The effect of different chemical and physical processing on the physicochemical and functional characterization of chitosan extracted from shrimp waste species of indian white shrimp. *Progress in Rubber, Plastics and Recycling Technology*. 2016; 32(1): 39–53.
- [15] Rojas J, Hernandez C, Trujillo D. Effect of the alkaline treatment conditions on the tableting performance of chitin obtained from shrimp heads. *African Journal of Pharmacy and Pharmacology*. 2014; 8(7): 211–219.
- [16] Percot A, Viton C, Domard A. Optimization of chitin extraction from shrimp shells. *Biomacromolecules*. 2003; 4: 12-18.
- [17] Naznin R. Extraction of Chitin and Chitosan from Shrimp (*Metapenaeus monoceros*) Shell by Chemical Method. *Pakistan Journal of Biological Sciences*. 2005; 8 (7): 1051- 1054.
- [18] Abdou ES, Nagy KSA, Elsabee MZ. Extraction and characterization of chitin and chitosan from local sources. *Bioresource Technology*. 2008; 99(5): 1359–1367.
- [19] Varun T, Senani W, Kumar N, Gautam M, Gupta R, Gupta M. Extraction and characterization of chitin, chitosan and chitooligosaccharides from crab shell waste. *Indian Journal of Animal Research*. 2017;(51): 1066-1072.
- [20] Alabaraoye E, Achilonu M Hester, R Biopolymer. (Chitin) from Various Marine Seashell Wastes: Isolation and Characterization. *J Polym Environ*. 2018; 26: 2207–2218.
- [21] Jang MK, Kong BG, Jeong YI, Lee CH, Nah JW. Physicochemical characterization of α -chitin, β -chitin, and γ -chitin separated from natural resources. *Journal of Polymer Science, Part A: Polymer Chemistry*. 2004; 42(14): 3423–3432.
- [22] Rinaudo M. Chitin and chitosan: Properties and applications. *Progress in Polymer Science*. 2006; 31(7): 603–632.
- [23] Younes I, Rinaudo M. Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs*. 2015; 13(3): 1133–1174.
- [24] Pillai CKS, Paul W, Sharma C. Chitin and Chitosan Polymers: Chemistry, Solubility and Fiber Formation. *Progress in Polymer Science*. 2009; 34: 641-678.
- [25] Islam M, Masum S, Rahman M, Shaikh A. Preparation of Glucosamine Hydrochloride from Indigenous Shrimp Processing Waste. *Bangladesh Journal of Scientific and Industrial Research*. 2011; 46(3): 375–378.
- [26] Younes I, Rinaudo M. Chitin and Chitosan Preparation from Marine Sources. Structure, Properties and Applications. *Marine Drugs*. 2015; 13(3): 1133-1174.