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Research Article

Changes in Quality Characteristics of *Crassostrea gigas* Oysters during Hypothermic Storage

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Abstract

Now oysters are one of the most common representatives of aquaculture grown worldwide. Finding reliable ways to assess the quality of oysters is an important task for breeding professionals and users of these products. The Food and Agriculture Organization considers microbiological testing and organoleptic parameters as mandatory tests for evaluation of the mollusks' safety. We believe these parameters in oysters are insufficient for unbiased evaluation of the condition of mollusks. In this research, we attempted to determine the most sensitive indices of oysters' viability during hypothermic storage under anoxia conditions. We investigated the oysters during 6 days of their storage in a household refrigerator at a temperature of 5°C. According to microbiological studies, during the experiment, we expected a rise in the total number of microorganisms. However, there was a paradoxical situation when at the end of the observations the total number of microorganisms decreased significantly, which contradicted the negative assessment of the state of oysters by organoleptic parameters. Flow cytometry studies also showed no correlation between the percentages of necrotized and apoptotic cells to organoleptic parameters. Studies have shown that on day 3 of storage, the oysters demonstrated a partial deterioration in organoleptic characteristics, which is clearly reflected in the content of volatile basic nitrogen in the tissues of mollusks, which characterizes the protein breakdown and accumulation of toxic nitrogen compounds. Thus, the content of volatile basic nitrogen in the tissues of oysters is the most sensitive and significant parameter in terms of biosafety of mollusks.

Keywords: *hypothermic storage, oysters, microbial flora, food quality, nitrogen*

INTRODUCTION

The growing aquaculture industry plays a crucial role in providing the world's population with food. Thus, the Food and Agriculture Organization reported in 2016 the total world production of food fish, mollusks and crustaceans reached 171 million tons, among which 17 million tons were mollusks. The development of breeding methods and expansion of the geography of mollusk consumption necessitate a more detailed and careful selection of assessment methods for nutritional suitability of these seafood.

Mollusks by their feed type are natural seawater filters. In the body of these animals, there are always microorganisms, which are identical to those of seawater. That is why in many countries of the world, where mollusks are used in food, the methods of bacteriological control of mollusk tissues have been developed and implemented as a guarantee of their quality and safety [5, 6, 16, 19-21]. The evaluation criteria for mollusks, and in particular oysters, are similar in different countries. To date it is mandatory to determine their total bacterial contamination or to examine the number of facultative-anaerobic microorganisms in colony-forming units (CFU per 1 g).

The intersection of different branches of science obviously produces the most amazing results of scientific research. In our work, we try to combine scientific cryobiological approaches and techniques for seafood storage to establish the methods for assessing the preservation of *Crassostrea gigas* oyster tissue during low-temperature storage (4... 5 ° C). The issue of safety and nutritional quality of mollusks is the reverse side of studying the morphology and function of biological specimens being under the negative effects of low temperatures. In cryobiological investigations, the general criterion to assess the effect of cold is the number of viable cells. Methods of light, electron microscopy, flow cytometry using the dyes and markers of different nature assist in non-biased assessment of the cell functioning after freeze-thawing or low-temperature storage [2, 9, 13, 14, 15, 22, 17]. The use of these methods is supported by examining physical and chemical metabolism of cold-exposed cells. In particular, in the reports [3, 11], the importance of the analysis of the content of proteins, lipids, carbohydrates, total content of nitrogenous substances in the bivalve samples after low-temperature storage to evaluate their quality has been emphasized. The research results on quality control and safety of mollusks as a food product have ensured the prospects of using physical and chemical methods. Thus, it was proven that such indices as the

content of TVB-N in the meat of mussels and medium pH allowed the revealing of the onset and development of biochemical processes during storage, which could be evidence of the product spoilage onset [8, 18]. The meat of marine fish and shellfish contains a large number of nitrogenous extracts, so it spoils faster than freshwater one. Because of the decomposition of protein by bacteria, the biogenic amines are formed, in particular histamine, which, in turn, can cause non-specific poisoning. During storage of perishable seafood, non-biased and sensitive methods of assessing the condition of raw materials are of paramount importance. Published reports confirm that in seafood after catching and during storage the autolytic and microbiological processes go in parallel. Autolysis disrupts the structure of muscle tissue and thereby promotes the penetration of bacteria into meat and initiates bacterial processes in it. To distinguish the autolysis and putrefaction is almost impossible [4, 6].

Of considerable practical interest are the studies on nutritional suitability of mollusks, which are stored under hypoxic conditions, i.e. in household refrigerators with no n aqueous medium because this is traditional storage method. Therefore, understanding the allowable time limits for safe hypothermic storage of oysters is vital in the field of catering.

The research aim was to validate the application of objective methods for assessing the preservation of *Crassostrea gigas* oyster tissue during low-temperature storage (4… 5 ° C).

MATERIALS AND METHODS

Mollusks

Crassostrea gigas oysters, trade name «Fin de Claire», grown *Marennes-Oléron*, (Charente-Maritime, France) were provided by the company (Oyster farm, Ukraine). The mollusks were delivered to the laboratory in a thermal container with ice. Live mollusks with intact and tightly closed shells, size №4, were selected for the experiment. The selected oysters were placed on ceramic pallets, covered with a damp cotton cloth and placed in a household refrigerator where they were stored for 1 week at a temperature of 4… 5 ° C. Every 3 days of storage, the preservation of mollusks was assessed. Within this research 15 samples of oysters were examined.

Sample preparation

In mollusks, a fragment of mantle and muscle tissue was separated, weighed, and homogenized using a glass homogenizer. The resulted homogenate was filtered through a nylon filter and centrifuged (5 min, 840g). The content of total protein was evaluated in supernatant. Measurements were performed with a biochemical analyzer ERBA CHEM 7 using the appropriate test system «Randox» (UK).

Flow cytometry

Flow cytometry (BD FACS Calibur, USA) with 7AAD, Annexin V FITC, and DCFH2-DA («Sigma-Aldrich» USA) dyes was used to examine the number of necrotic cells, percentage of cells that had progressed to apoptosis, as well as we examined the presence of ROS cells, respectively. Cells for this study were obtained from a fragment of muscle tissue by enzymatic disaggregation with collagenase.

Sensory analysis

Organoleptic quality of oysters was examined by a sensory assessment, which was performed by the expert evaluation on a five-point scale. The overall appearance, signs of vital activity, body color and consistency, aroma, and taste were scored.

Chemical analysis

To test the pH, the oyster tissue extract was prepared in 1:4 ratio. With this aim to 10 g of crushed oyster tissue there was added 40 ml of distilled water and extracted for 15 minutes. After that, the extract was paper-filtered and the pH in liquid fraction was determined using a potentiometer.

Non-protein nitrogen content was determined as follows. To a 250… 300 ml conical flask 10.00 ml of oyster tissue extract prepared as described above was pipetted, there was added 40 ml of distilled water and 3 drops of 1% solution of phenolphthalein. The extract was neutralized with 0.1 N sodium hydroxide solution until a pale pink color appeared. Then, 10 ml of a 37% solution of phenolphthalein-neutralized formalin was added to the flask and again titrated with 0.1 N sodium hydroxide solution until a pale pink color appeared. The content of TVB-N (mg / 10 ml of extract) was calculated by the formula:

$$X = 1.4\ V$$

where V – is the volume of 0.1 N sodium hydroxide solution spent on the second titration, ml.

The mass fraction of water in the tissue of oysters was determined using the All-Union Standards 7636-85. A portion of the test sample (1.5-2 g) was weighed to the nearest 0.001 g, placed in a dried and calibrated box with a glass rod, with which a portion of the product in the box in an even thin layer was distributed. The box was closed with a ground lid, weighed on analytical balances and dried in an oven at a temperature of 100… 105°C to constant weight. The mass of the test portion to be dried was considered constant when the difference between the last two tissue specimens did not exceed 0.001 g. The mass fraction of water (%) was calculated by the formula:

$$W = \frac{m_1 - m_2}{m_1} \cdot 100\,\%$$

where m_1 - mass of the sample of oysters before drying, g; m_2 - mass of the oyster sample after drying, g. Arithmetic mean of 3 parallel tests was assumed as a final result.

Microbiological analysis. Microbiological studies were performed in accordance with the National Standards of Ukraine 7821: 2015 Live gastropods. Specifications and All-Union Standards 10444.15-94 Food Products. Methods for determining the number of mesophilic aerobic and facultative-anaerobic microorganisms (Food. Methods for determining the number of mesophilic aerobic and facultative-anaerobic microorganisms).

Statistical analysis

These results were statistically processed according to Student's criterion with 0.95 reliability.

RESULTS AND DISCUSSION

Flow cytometry results

At early stages of the research the condition of *Crassostrea gigas* oyster muscle tissue cells were assessed by flow cytometry. However, the data obtained by us contradicted with the organoleptic parameters, which indicated a gradual deterioration of the tissues of mollusks during hypothermic period of storage. It is logical to hypothesize that the number of necrotic cells, the percentage of cells that underwent apoptosis, and the level of ROS in the cells should have increased with prolonging the shelf life, but the results of flow cytometry demonstrated an opposite. The values obtained at the stage of storage for 6 days were of an oscillatory nature,

which may indicate a restructuring of the adaptive processes of mollusks, being in unfavorable conditions for a long time. Figure 1 shows the results of preservation of *Crassostrea gigas* oyster cells, using the 7AAD dye. It has been suggested that the fluorescent markers 7AAD, annexinV and DCF cannot be used in future studies when assessing the seafood quality.

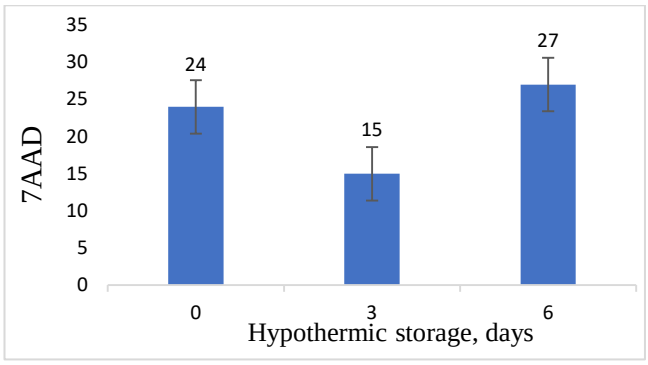

Figure 1: Number of necrotic cells during experiment, %.

It has been reported [2, 13] those certain fluorescent markers are not reliable for assessing the condition of mollusk cells. In particular, annexin V does not provide an accurate result when used to assess early apoptosis. When using DNA dyes such as 7AAD, the detection of the true number of cells is complicated by the fact that mechanically damaged cells and isolated nuclei can be misinterpreted by the fluorescent dye as individual cells. Therefore, we concluded that the method we used to assess the preservation of mollusk cells requires significant rethinking and refinement. The lack of adequate oyster cell status indices using flow cytometry has led us to look for more objective correlates of mollusk survival.

Results of chemical analysis

Most metabolic processes, the course of which requires significant energy expenditure, including the protein synthesis, are known to be inhibited by adverse factors (hypothermia, anoxia) [10]. Analysis of the content of total protein in the tissue of mollusks revealed that from day 1 to day 6 of hypothermic storage, the content of total protein in the tissue of mollusks significantly increased Figure 2.

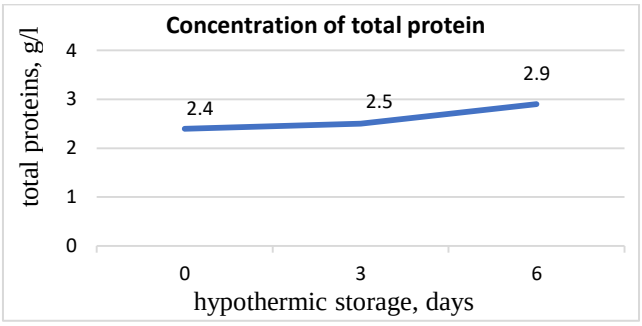

Figure 2: Change in total protein of oyster tissue homogenate after hypothermic storage

Such changes may be an evidence of autolysis in mollusk tissues and are consistent with the organoleptic characteristics of oysters.

It is well known that proteins are mostly affected in autolysis, the main typical indices of autolysis in seafood (spoilage scoring) are volatile nitrogenous bases, ammonia and trimethylamine [4].

The paper [11] described the food suitability of mussels during storage in the refrigerator at 4°C for 6 days. The authors pointed out that sensitive chemical indicators of the quality and spoilage of mussels are the products of decay of nitrogen-containing compounds, in particular total volatile basic nitrogen (TVB-N), trimethylamine (TMA-N) and indole. This research emphasizes the value of examining the chemical indices as the indicators of the initial stage of mollusks spoilage. Accumulation of protein degradation products may be the most sensitive and objective index of adverse changes in mollusk tissues during long-term storage. An important point is that the change in physical and chemical parameters occurs before their deterioration by organoleptic characteristics. The use of only organoleptic studies does not fully address the quality of mollusks.

Therefore, in our further investigations we examined the preservation rate of oyster tissues according to the following indices: pH, amount of non-protein nitrogen and water content in oyster meat during hypothermic storage. The findings are shown in Table 1.

Table 1: Physical and chemical assessments of preservation rate of oyster tissues during hypothermic storage

Storage term, days	pH	Content of total volatile basic nitrogen in oyster meat (mg/10 ml of extract)	Water content in oyster meat (%)
0	6.39±0.01	2.35±0.02	81.5±0.4
3	6.46±0.01	3.27±0.03*	73.9±0.4
6	6.49±0.01	3.36±0.03*	79.9±0.4

Note: * indices significantly exceeded the upper limits of the norm

According to the data obtained after 3 days of hypothermic storage, the content of TVB-N in oyster meat was higher than 3.00 mg / 10 ml of extract, which is typical for stale meat. At the same time, the water content in oyster meat changed slightly.

Results of organoleptic analysis

Changes in organoleptic quality of oysters during the experiment completely correlated with those in the amount of non-protein nitrogen in the meat of mollusks. The results of the study of organoleptic characteristics of oyster meat during hypothermic storage are presented as a profilogram in Figure 3.

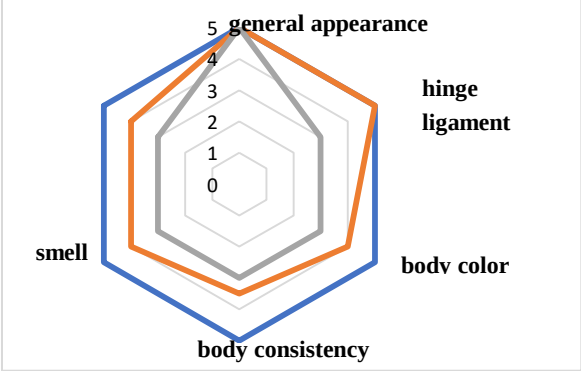

Figure 3: Scores of oyster meat during hypothermic storage: — 0 days; — 3 days; — 6 days

According to the results of organoleptic analysis, it was found that during the shelf life the appearance of oysters remained unchanged: the surface of the wings was clean, without silt and sand; on the surface of the wings there are small remnants of balanus shells. Signs of vital activity begin to deteriorate on day 6: the wings of mollusks were not closed very tightly. The color changed from light gray to beige on day 3, the body of the mollusk became matte. On day 6 the oyster body was of a yellowish color (Fig. 4). The body consistency also noticeably deteriorated from day 3. Smell and taste became unpleasant starting from day 6.

a) b) c)

Figure 4: Appearance of oysters in different storage periods: a) fresh oysters at the experiment beginning; b) after 3 days; c) after 6 days.

Thus, a comparative assessment of the organoleptic characteristics of oyster meat shows the instability of the mollusk properties during the studied shelf life.

Results of microbiological tests

The next stage of the research was the study of microbiological quality indices, namely the total bacterial count. According to the norm the total bacterial count of oysters for culinary production should not exceed $1 \cdot 10^4$ CFU. Total bacterial counts were done in accordance with accepted standards [1, 6, 7]. With this aim the corresponding solutions of oyster's meat were prepared: 10^{-3}; 10^{-4}, which were introduced by 1 cm^3 into sterile Petri dishes and poured with 15 cm^3 of cooled down to 45 ˚C MPA (meat peptone agar). Dishes with cultures were kept in a thermostat at a temperature of (32 ± 1) ° C for 72 hours. The results were evaluated by counting the total number of CFUs. The experiment was performed in parallel in three samples on days 3 and 6 of storage. The results of examining the total bacterial count in oyster samples are shown in Table 2.

Table 2: Total bacterial count of oysters, CFU/cm^3 at various storage terms

Sample №	Number of CFU/cm^3 at storage term of:	
	3 days	6 days
1	10,000	1,000
2	21,000	1,000
3	46,000	1,000

Table 3 demonstrates that the rate of total bacterial count decreases significantly on day 6 of storage of oysters, that hypothetically can be explained by the effects of digestive enzymes.

The data obtained on the storage of oysters in an anhydrous medium indicate that conventional microbiological methods do not provide an adequate assessment of the food safety of mollusks.

This method of storage significantly reduces a possible contamination by microorganisms via water. In the tissues of mollusks the autolytic processes are dominating. This is confirmed by our results on an increased content of volatile basic nitrogen in the tissues of oysters during hypothermic storage. It should be noted that according to the Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004, fishery products entering the market must be tested for the content of total volatile nitrogenous base (TVB-N) [12]. Also, similar requirements are imposed on fishes and their products in other countries. For example, this applies to the State Standard of the People's Republic of China GB 18406.4-2001 [19]. However, in all countries without exception, these indices are not considered as a criterion for assessing the safety of mollusks.

Comparison of the results obtained by different methods of assessing the preservation of mollusk tissue during hypothermic storage showed that the most sensitive to negative changes in the quality of mollusks are the TVB-N indices, which convincingly correlate with organoleptic parameters and allow to qualitatively evaluate the preservation of oyster's tissue as a food product. The water content of oyster meat was also somewhat adjusted for the changes in organoleptic quality of mollusks.

CONCLUSION

We compared the results obtained by microbiological, chemical and organoleptic tests, as well as flow cytometry analysis, which were used to assess the nutritional quality of oysters. It is very likely that the limited data in the published reports and lack of studies on the use of the TVB-N in assessing the food safety of mollusks demonstrate its undervaluation.

The findings will be helpful in developing the international standards for assessing the nutritional suitability of mollusks.

Conflicts of Interest:

Authors disclose no personal or financial relationships that could be viewed as potential conflicts of interest in relation to the publication on manuscript.

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