УДК 664.959.5:597.555.51

The use of protein hydrolysate from fish waste as part of microbiological culture media

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Article info

Received 21.03.2024;

accepted 08.05.2024

Key words:

fish protein hydrolysate, fish wastes, · recycle fish materials, culture medium, · coefficient of productivity of culture medium, identifying test microorganisms

Abstract

The need to involve in the processing of significant amount of secondary protein-containing raw materials formed during fish cutting, on the one hand, and on the other hand, the shortage of a source of the protein component of microbiological nutrient media determines the relevance of the work. The goal was to study the possibility of using fish protein hydrolyzate obtained by enzymatic hydrolysis from secondary fish raw materials in microbiological nutrient media. Microbiological and physicochemical methods were used to conduct research. When optimizing the developed algorithm for the fermentation process, a mathematical model of the experiment was used with a minimum number of experiments. An algorithm has been developed for obtaining fish protein hydrolyzate from meat and bone waste from cutting pelagic fish (cod). The hydrolysis parameters were optimized: the concentration of the enzyme preparation is 1.33 % of the total mass of waste, the duration of the fermentation process – 3 hours. The characteristics of the resulting fish hydrolyzate are determined: the mass fraction of total nitrogen – 13 %, amine – 3.6 %, water – 4.6 %, sodium chloride – 2.7 %, fat – 0.3 %. An experimental fish hydrolyzate has been studied as a source of protein nitrogen in accordance with the formulation of a microbiological medium used for counting when growing colonies of microorganisms. The effectiveness of the prepared nutrient media was assessed by comparing the performance coefficients of the experimental and control nutrient media, as well as by identifying test microorganisms grown on the experimental medium. It was established that the growth pattern of test cultures both on the studied nutrient medium and on the control medium was almost the same. Test microorganisms retained their biochemical, morphological and cultural characteristics. The research results showed the possibility of using fish protein hydrolyzate obtained by enzymatic hydrolysis from secondary fish raw materials in microbiological nutrient media.

For citation

Zhivliantceva, I. V. et al. 2024. The use of protein hydrolysate from fish waste as part of microbiological culture media. *Vestnik of MSTU*, 27(3), pp. 294–301. (In Engl.) DOI: https://doi.org/10.21443/1560-9278-2024-27-3-294-301.

Использование белкового гидролизата из вторичного рыбного сырья в составе микробиологических питательных сред

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Информация о статье

Поступила в редакцию 21.03.2024;

принята к публикации 08.05.2024

Ключевые слова:

рыбный белковый гидролизат, вторичное рыбное сырье, питательные среды, коэффициент производительности питательной среды, идентификация тест-микроорганизмов

Реферат

Необходимость вовлечения в переработку значительного количества вторичного белоксодержащего сырья, образующегося при разделке рыбы, с одной стороны, и с другой – дефицит источника белковой составляющей микробиологических питательных сред определяет актуальность работы. Целью являлось исследование возможности использования в составе микробиологических питательных сред рыбного белкового гидролизата, полученного методом ферментативного гидролиза из вторичного рыбного сырья. Для проведения исследований применялись микробиологические и физико-химические методы. При оптимизации разработанного алгоритма процесса ферментации использовали математическую модель эксперимента при минимальном числе опытов. Разработан алгоритм получения рыбного белкового гидролизата из мясокостных отходов от разделки пелагических рыб (трески). Оптимизированы параметры гидролиза: концентрация ферментного препарата – 1,33 % к суммарной массе отходов, продолжительность процесса ферментации – 3 ч. Определены характеристики полученного рыбного гидролизата: массовая доля общего азота составила 13 %, аминного – 3,6 %, воды – 4,6 %, хлористого натрия – 2,7 %, жира – 0,3 %. Опытный рыбный гидролизат исследован в качестве источника белкового азота в соответствии с рецептурой микробиологической среды, используемой для подсчета при выращивании колоний микроорганизмов. Эффективность приготовленных питательных сред оценивали путем сравнения коэффициентов производительности опытной и контрольной питательной среды, а также методом идентификации выросших на экспериментальной среде тест-микроорганизмов. Установлено, что характер роста тест-культур как на исследуемой питательной среде, так и на контрольной практически одинаковый. Тест-микроорганизмы сохранили свои биохимические, морфологические и культуральные признаки. Результаты исследований показали возможность использования в составе микробиологических питательных сред рыбного белкового гидролизата, полученного методом ферментативного гидролиза из вторичного рыбного сырья.

Для цитирования

Живлянцева Ю. В. и др. Использование белкового гидролизата из вторичного рыбного сырья в составе микробиологических питательных сред. Вестник МГТУ. 2024. Т. 27, № 3. С. 294–301. DOI: https://doi.org/10.21443/1560-9278-2024-27-3-294-301.

Introduction

The fundamental "tool" in microbiology is culture media. They are used for research purposes, selection and research of isolated types of microorganisms, creation of new vaccines and medicines, for other purposes related to pharmaceuticals, biology, and medicine. Today on the market there is a very large number of liquid and powder media for microbiology from the world's largest manufacturers "Pronadisa" laboratorios Conda, S.A. (Spain), Merck (Germany), bioMerieux (BioMerieux, France), Neogen (America). They have the appropriate quality certificates, but their cost is quite high. In this regard, the development of technologies for the production of import-substituting culture media is urgent. The main component of most culture media are the products of protein hydrolysis of plant and animal origin. Until now, pancreatic hydrolysate of fish meal (State research center for applied biotechnology and microbiology, Russia, Obolensk), enzymatic hydrolysate of casein (Pronadisa Conda, Spain), casein and soy peptones (Merck, Germany), pancreatic hydrolysate of casein have been widely used as protein bases, papain hydrolysate of soybeans (BioMerrier, France), gelatinous peptone and meat extract (Neogen, America), etc. However, due to the cessation of fishmeal production as well as the introduction of sanctions on the import of imported raw materials, there is a shortage of this type of product. For the production of protein hydrolysate – a source of nitrogen in the composition of microbiological culture media – we used an alternative source of raw materials: recycle fish materials, namely, meat and bone waste from cutting pelagic fish. Fish waste is an important underestimated reserve of raw materials. Research into the use of protein from fish waste as a component of culture media has been carried out by the research team for a number of years since 2016. Waste from cutting pelagic fish species on fillets makes up 57–64 % on average of the mass of fish received for cutting, and the share of musculoskeletal waste accounts for up to 30–35 % (*Derkach et al., 2017*; *Дровянинова и др., 2015а*). At the same time, they contain a significant amount of complete animal protein. Analysis of information sources (*Дровянинова и др., 2015б; Касьянов, 2015; Максимюк и др., 2009*) had showed that it was the rationality of the use of recycle materials and waste that would reduce the raw material shortage in the production of microbiological nutrient media. The involvement of this source of raw materials in processing is a promising direction for the development of the most important branch of modern agriculture (*Ломовцева и др., 2019*).

Materials and methods

The purpose of the work was to study the possibility of utilization a hydrolysate from fish waste as a protein component of microbiological media. To achieve this goal, the following tasks were solved: to optimize the previously developed algorithm for the fermentation of fish waste; to study the physicochemical and biochemical parameters of a prototype of fish protein hydrolysate; to evaluate the quality of culture media used for growing microorganisms prepared using a prototype hydrolysate.

In the developed algorithm for obtaining a prototype fish protein hydrolysate, the objects of study were musculoskeletal waste of cod fillets (cod caught by the "Public Joint-Stock Company Murmansk Trailing Fleet" in the fishing areas of the Barents Sea), an enzyme preparation – protosubtilin G3X – a product of the activity of bacteria of the *Bacillus subtilis* strain (Sibbiopharm, Russia), an experimental sample of fish protein hydrolysate obtained in the process of enzymatic hydrolysis.

Physical and chemical indicators such as content of water, fat, proteins, amine nitrogen and minerals were determined by standard methods GOST 7636-85 "Fish, marine mammals, marine invertebrates and products of their processing. Analysis methods"[1]. The protein content was determined by the Kjeldahl method using equipment consisting of two elements: BLOCK-DIGEST 12 for sample mineralization and an automatic distillation unit PRO-NITRO A (J.P. Selekta, Spain). The fat content was determined by the Soxhlet method using a Selecta DET-gras extractor (Spain).

The degree of hydrolysis was defined by the calculation method as the ratio of the mass fraction of amine nitrogen to the mass fraction of total nitrogen in the hydrolysate (*Производство..., 1990*).

For establishing the optimal parameters of enzymatic hydrolysis two factors rotatable compositional plan was used. Mathematical data processing was carried out using the DataFit program, version 9.1 (*Решетников, 2000*).

The experiments were carried out in triplicate, the data obtained were subjected to one-way analysis of variance (ANOVA) using Origin Pro 8.0. Differences between the means were considered significant at $p < 0.05$.

We prepared culture media using the obtained fish protein hydrolysate according to the formulation of the control culture medium. The pancreatic fishmeal hydrolysate was replaced with a prototype fish protein hydrolysate. As a control medium, we used "Nutrient agar for the cultivation of microorganisms GRM" (manufactured by State research center for applied biotechnology and microbiology, Russia, Obolensk).

To control the efficiency of culture media, test microorganisms with stable characteristics were used.

The work used microorganisms with stable characteristics, which are the causative agents of the most dangerous food poisoning.

[1] Information on regulations and GOSTs is presented in the Appendix.

Test microorganisms were obtained from specialized collections: *Staphylococcus aureus* ATCC 25923 *ROSENBLACH 1884*; *Salmonella enteriditis* No. 5765 (*ex Kauffmann & Edvards 1952*) *le minor & Popov 1987*.

For the prepared culture media: control and test (containing a test sample of fish protein hydrolysate), coefficient of productivity of culture medium was calculated according to GOST ISO 11133-2016 "Microbiology of food products, animal feed and water. Preparation, production, storage and determination of the working characteristics of nutrient media". After cultivation on the studied culture media, biochemical signs during the identification of microorganisms were determined using test systems (Diagnostic kit No. 2, Scientific and Production Association "Microgen", Russia).

Results

A prototype of fish protein hydrolysate was obtained by enzymatic hydrolysis of cod musculoskeletal waste. As an enzyme preparation, we used protosubtilin G3X – a product of the activity of bacteria of the *Bacillus subtilis* strain (Sibbiopharm, Russia). The proteolytic activity of the enzyme is 560.77 μmol TYR/g.

One of the quality parameters of fish protein hydrolysate is the degree of hydrolysis determined in various ways. The most common indicator is the ratio of the mass fraction of amine nitrogen to the mass fraction of total nitrogen in the hydrolysate.

The degree of hydrolysis of the obtained fish protein hydrolysate is 28.06 % (*Куранова и др., 2016*). According to the literature data the degree of hydrolysis of the most promising enzymes – bromelain and papain – is 23.74 %.

In addition, the choice of the enzyme was due to its lower cost compared to other enzymes, for example, trypsin, pancreatin, pepsin, bromelain and papain, etc., which are used in the production of industrial fish protein hydrolysates.

When optimizing the fermentation parameters, the dosage of the enzyme preparation was varied in relation to the mass of the hydrolyzed raw material (X_1, %) and the duration of exposure (X_2, hour) at the previously specified temperature of 45 °C. The numerical characteristic of the value of the achieved degree of hydrolysis (Y, %) was chosen as an optimized parameter. We used a rotatable composite plan (*Живлянцева и др., 2018*) for a two-factor experiment (Table 1).

Table 1. Results of experimental data processing on optimization
of the fermentation stage of hydrolysis of cod waste
Таблица 1. Результаты обработки данных экспериментов
по оптимизации стадии ферментации гидролиза отходов трески

Parameter	1	2	3	4	5	6	7	8	9
X_1, %	0.6	0.6	1.5	1.5	1.05	1.69	0.41	1.05	1.05
X_2, hour	2	5	2	5	3.5	3.5	3.5	1.38	5.62
Y, %	23.5	23.9	26.5	27.0	27.5	27.6	22.8	24.3	27.6

As a result of processing experimental data the following equation describing the influence of the studied factors on the optimization option was received (*Живлянцева и др., 2018*)

$$Y = ax_1 + bx_1^2 + cx_2 + dx_2^2 + ex_2^3, \quad (1)$$

where Y – the value of the achieved degree of hydrolysis, %; x_1 – the dosage of enzyme preparation, %; x_2 – the duration of hydrolysis, hour; a, b, c, d, e – the regression coefficients: a = 17.08; b = –6.42; c = 14.14; d = –3.80; e = 0.32.

Fisher's criterion for this equation is 80.84, it means that with the given confidence level (0.99), the regression equation reliably describes the change in the optimization parameter depending on the change in factors X_1 and X_2.

To find the optimal values of the factors X_1 and X_2 determining the optimal parameters of the hydrolysis process, we used the methods of mathematical processing (differentiation).

The values of these optimal factors are as follows: X_1 (concentration of the enzyme preparation) – 1.33 % by weight of raw materials; X_2 (duration of the hydrolysis process) – 3 hours (*Живлянцева и др., 2018*). Considering the research carried out, an algorithm for obtaining a fish protein hydrolysate has been developed.

For further research, we used a hydrolysate prepared according to the developed algorithm, considering the optimized fermentation conditions. The quality indicators of the prototype fish protein hydrolysate are presented in Table 2.

Fish protein hydrolysate is an amorphous, light yellowish powder with a weak mushroom aroma. It has the ability to emulsify, foaming, when dissolved in water gives opalescent solutions, which confirms the preservation of the properties of the native protein in the product. Studies of the biological value of the product had found that the hydrolysate contained all protein amino acids, including essential ones, the minimum amount contains tryptophan (4.0 mg/g protein), the maximum amount contains hydroxyproline (150.6 mg/g protein) (*Куранова и др., 2016*).

Table 2. Quality indicators of a fish protein hydrolysate prototype
Таблица 2. Показатели качества опытного образца рыбного белкового гидролизата

The name of indicators	Value
Consistence	Amorphous homogeneous powder
Colour	Light beige
Aroma	Typical for this type of product
Active acidity, pH	6.75 ± 0.10
Moisture content, %	4.60 ± 0.26
Mass fraction of amine nitrogen, %	3.60 ± 0.09
Mass fraction of total nitrogen, %	12.97 ± 0.04
Mass fraction of fat, %	0.26 ± 0.02
Mass fraction of sodium chloride, %	2.70 ± 0.12

The experimental sample of fish protein hydrolysate received according to an optimized fermentation mode was used to make a culture medium in a control nutrient agar formulation, in which pancreatic fish meal hydrolysate is used as a nitrogen source. In the experimental culture medium, the pancreatic fishmeal hydrolysate was completely replaced by the experimental fish protein hydrolysate. As a control, we used "Nutrient agar for the cultivation of microorganisms, dry GRM". The formulations of the test and control culture media are presented in Table 3.

Table 3. Recipes of culture media
Таблица 3. Рецептуры питательных сред

Name	Amount, g (ml)/1 l of distilled water
Nutrient agar for the cultivation of microorganisms, dry GRM	
Peptone for bacteriological culture media	12
Fishmeal Pancreatic Hydrolysate	12
Sodium chloride	6
Agar	10
Study culture medium based on a prototype fish protein hydrolysate	
Peptone for bacteriological culture media	12
Prototype fish protein hydrolysate	12
Sodium chloride	6
Agar	10

To control the quality of culture media, test microorganisms with stable characteristics was used.

The prepared culture media were intended for counting the column of microorganisms. To control the quality of the prepared culture media, the coefficient of productivity of the culture medium was calculated.

The productivity of the culture medium is the degree of growth of the target microorganism on the culture medium under certain conditions. Sowing test-cultures on the control and test media was performed by direct inoculation. Crops were incubated in a thermostat at a temperature of 37 ± 1 °C for 18–24 hours. Microorganisms were counted using GOST ISO 7218-2015 "Microbiology of food and animal feed. General requirements and guidance for microbiological examinations".

Comparative characteristics of the growth performance of test microorganisms on the studied culture medium based on fish protein hydrolysate and nutrient agar GRM are presented in Table 4.

Table 4. The growth form of test microorganisms on the studied culture medium based on fish protein hydrolysate and nutrient agar GRM
Таблица 4. Сравнительная характеристика роста тест-микроорганизмов на исследуемой питательной среде на основе рыбного белкового гидролизата и питательном агаре ГРМ

Tested culture media	Dilution	*Staphylococcus aureus* ATCC 25923	*Salmonella enteriditis* № 5765
Tested culture medium based on a prototype fish protein hydrolysate (N_c)	10^{-6}	1.3×10^{9}	3.8×10^{9}
Nutrient agar for the cultivation of microorganisms, dry GRM (N_c)	10^{-6}	1.3×10^{9}	3.5×10^{9}

The productivity of culture medium was assessed as the degree of growth of the target microorganism on the culture medium when compared with culture agar for the cultivation of microorganisms GRM (GOST ISO 11133-2016) under certain conditions and was calculated by the formula

$$P_R = N_c / N_0, \quad (2)$$

where P_R – the coefficient of productivity of culture medium; N_c – the arithmetic mean of the number obtained on the culture medium subjected to the test (for example, the number of colonies in plates); N_0 – the arithmetic mean of the amount obtained on a certain control culture medium.

The coefficients of productivity of culture medium are presented in Table 5.

Table 5. Coefficient of productivity of culture medium
Таблица 5. Коэффициенты производительности

Name	*Salmonella enteriditis* № 5765	*Staphylococcus aureus* ATCC 25923
Tested culture medium based on a prototype fish protein hydrolysate	1.00	1.09
Nutrient agar for the cultivation of microorganisms, dry GRM	1	1

Description of the obtained results

The results are considered valid when the following conditions are met:

– a positive quantitative result (target bacterial growth) must be obtained for each plate;

– each individual declared result falls within the standard assay range (no more than 100 colonies for methods with filtration and no more than 150 colonies for surface methods). P_R should be at least 0.70 when comparing non-selective media with non-selective control media according to GOST ISO 11133-2016.

The biochemical characteristics of colonies of microorganisms grown on the studied media were investigated. The work used microorganisms with stable characteristics, which are the causative agents of the most dangerous food poisoning.

The most common analogue of fish protein hydrolysate is pancreatic fish meal hydrolysate. According to GOST 29311-92 "Pancreatic hydrolysates for bacterial culture media. General specifications" the main defining indicators of this type of products are the mass fraction of total and amine nitrogen, confirming the presence of the required number of nitrogenous compounds necessary for the life of microorganisms. For pancreatic fish meal hydrolysate, which is used for the preparation of culture media, the value of the mass fraction of amine nitrogen should be at least 0.3 %, total nitrogen – at least 0.75 %. According to TU 480-00001927 "Pancreatic hydrolysate of fish meal. Specifications" the mass fraction of amine nitrogen must be at least 2.8 %, total nitrogen – at least 8 %. According to Table 2, it can be seen that the corresponding values of the experimental fish protein hydrolysate are higher (3.60 and 12.97 %, respectively). It indicated the sufficiency of nitrogenous nutrients in the sample under study.

Microbiological studies have established that the nature of the growth of test cultures on the studied culture medium using a test sample of fish protein hydrolysate as a protein component and control nutrient agar are identical.

Discussion

Coefficient of productivity of culture media has shown an efficiency of microbiological culture media. The studied culture medium showed high productivity ratio (P_R = 1, P_R = 1.09) when cultivating *Salmonella enteriditis* No. 5765 and *Staphylococcus aureus* ATCC 25923, respectively, which was at the level and even slightly higher than the performance values of the control medium and significantly exceeded the requirements for productivity (P_R more than 0.7) established in GOST ISO 11133-2016.

The test microorganisms after cultivation on the studied nutrient media based on fish protein hydrolysate retained their biochemical characteristics during the identification of microorganisms using test systems (Scientific and Production Association "Microgen", Russia).

Bacteria of the genus *Salmonella* retained their gram-negative Gamma colour, the growth pattern on three-sugar ferruginous agar did not change (they ferment glucose and do not ferment lactose and sucrose, form hydrogen sulphide). Bacteria of the genus *Salmonella* do not form acetoin (Voges-Proskauer reaction is negative). *Salmonella* bacteria ferment mannitol. Agglutination (presence of O-antigens) is manifested in the form of sticking of the bacterial mass and full or partial clarification of the liquid (according to GOST 31659-2012 "Food products. Method for detecting bacteria of the genus *Salmonella*").

Bacteria of the genus *Staphylococcus aureus* retain a gram-positive Gram colour, give a positive reaction to catalase, form acetoin and ferment maltose under aerobic conditions. They have the ability to coagulate rabbit

blood plasma (according to GOST 31746-2012 "Food products. Methods for detecting and quantifying coagulase-positive staphylococci and *Staphylococcus aureus*").

Colonies of microorganisms retain their morphological and cultural properties. Therefore, for these test cultures, nutrients were sufficient on all studied media. The results of microbiological studies confirm the possibility of using fish protein hydrolysate obtained in the process of hydrolysis from cod musculoskeletal waste as a component of general-purpose nutrient media for counting colonies of microorganisms.

Conclusion

Thus, as a result of the conducted research:

– the parameters of the fermentolysis are optimized: duration – 3 hours, enzyme concentration – 1.33 %;

– there are determined chemical and biochemical parameters of the prototype fish protein hydrolysate (mass fraction of amine nitrogen must be at least 3.6 %, total nitrogen – at least 13.0 %), which meet the requirements for the indicators of protein components of nutrient media;

– the quality of nutrient media using a prototype fish protein hydrolysate as a protein component to the operational criteria of GOST ISO 11133-2016 is established;

– the developed fish protein hydrolysate can be recommended for the preparation of general-purpose culture media for counting colonies of microorganisms.

It has been also determined that in the culture medium "Nutrient agar for the cultivation of microorganisms dry GRM" the developed product could completely replace "Pancreatic hydrolysate of fish meal".

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The study was carried out through proactive R&D of Murmansk Arctic University "Development and improvement of technologies for the production of food, feed and technical products of a wide range from animal and plant raw materials of the Arctic region".

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Appendix

Regulatory documents used in the paper

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