Simulation of fermentolysis of secondary meat and bone raw materials

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Abstract. The study is of current relevance due to an underestimated potential of secondary meat and bone raw materials, which contain valuable bioactive substances that can be used in food, feed, cosmetic and other products. Hydrolyzing of extracting protein, fat and mineral substances from such raw materials is a promising method. The study aimed to investigate and simulate fermentolysis of highly mineralized collagen-containing raw meat with proteolytic enzymes. The experiments were carried out on beef tubular and rib bones using Alcalase 2.5 L, Protamex and Protosubtilin G3x enzymes. The efficiency of protein hydrolysis was assessed with regard to the parameters of formol titratable nitrogen, dry matter, titratable acidity, which were determined in aqueous solution of the fermentolysat. The quality indicators of the hydrolysis products were assessed by standard physicochemical methods. The data obtained were processed by methods of mathematical statistics. A mathematical model of fermentolysis of meat and bone raw materials was developed based on the analysis of graphical dependences of formol-titrated nitrogen parameters and dry matter on the enzyme preparation dosage and process duration. The models can be used to assess the decomposition level of proteins and their water extraction for various types and dosages of enzyme preparations, and to determine rational duration of fermentolysis. The model can be used as a basis to reveal the effect of the main factors on the degree of hydrolysis of proteins in meat and bone raw materials and to control the process of fermentolysis.

1. Introduction
The meat industry in the Russian Federation has currently a high economic and technological reserve in the form of 1 million tons of secondary raw materials, which are underutilized annually, including meat and bone raw materials (tibiae of cattle, rib bones, bone meal, etc.). In the ideal case, these raw materials are processed into feed products or sold cheap without processing. However, chemical composition of meat and bone raw materials is of high biological potential since the bulk of dry matter includes valuable bioactive substances (BAS) of animal origin. These are proteins, minerals and fats that can be extracted and used as premium dietary supplements for food, feed, pharmaceutical and...
other purposes. This requires appropriate technological solutions based on biotechnological methods and mathematical models to control technological parameters and modes [1, 2].

At the Department of Food Biotechnology, Kaliningrad State Technical University, a hydrolysis method has been developed for processing highly mineralized collagen-containing tissues (bones, scales, heads, feathers), which includes secondary meat and bone raw materials (tibiae and thigh bones of animals). The method involves enzymatic or high-temperature treatment, or a combination of these processes followed by fractionation, extraction, isolation and conservation of the main groups of organic matter. The method is employed with regard to specific features depending on the type of the raw material [3–6].

Enzymatic hydrolysis with proteolytic enzymes is the most appropriate method for processing of secondary collagen-containing raw meat to obtain the main and most valuable fraction – protein products (low molecular weight proteins, peptides, and amino acids). Enzymatic hydrolysis allows obtaining high quality hydrolysates with preserved features of amino acids without by-products. However, this method is virtually not used for highly mineralized meat and bone raw materials since it is a time-consuming process due to high strength of the connective tissues formed by procollagen proteins when combined with minerals. This requires efficient highly active enzymes with the specificity corresponding to that of highly mineralized substrates. Virtually no data on fermentolysis of this raw material are available in the scientific technological literature, which mainly reports on its processing with enzyme preparations after preheating of raw materials [4, 7–11] to obtain collagen substances for food, feed and technical purposes [12–17]. At the same time, studies devoted to fermentolysis of mineralized animal tissues to produce active peptides with various types of bioactivity (antioxidant, antihypertensive, etc.) are increasing in number [18–22].

To study the process of fermentolysis of meat and bone raw materials and the use of its products for various purposes, it is rational to reveal dependencies in graphic and/or mathematical form. For these studies, proteolytic enzymes (enzyme preparations – EP) of collagenase specificity should be used. Even the most active of them, however, do not fully decompose high molecular weight proteins, since they are consumed in the enzymological process, and the reactions stop [1, 4, 5].

The aim of this study was to simulate fermentolysis of beef meat and bone raw materials with various enzymes to reveal reliable dependences at this technological stage to extract valuable organic bioactive substances.

Mathematical models and graphical dependencies are required to control fermentolysis, determine the most rational enzyme preparations and their quantities, and to predict the duration and depth of the process, yield and quality of target products.

2. Methods
A series of experimental studies on enzymatic processing of secondary meat and bone raw materials was performed at the Center for Advanced Technologies of Protein Use, Department of Food Biotechnology, Kaliningrad State Technical University (KSTU, Kaliningrad)

The raw materials used in the experiments included beef tibiae with heads and beef ribs provided by Golubevsky meat-packing plant LEAR, and beef ribs from the slaughterhouse in the village of Dobroe. The raw materials were provided fresh and frozen and were stored until the experiment at temperature not exceeding minus 18 °C.

Three proteolytic EPs of broad specificity used as enzyme preparations performed well in hydrolysis during processing of collagen-containing raw materials: Alcalase 2.5 L and Protamex (Danish company Novozymes), EP of domestic production Protosubtilin G3x (OOO PO Sibbiopharm). The characteristics of the EPs are shown in Table 1.
Table 1. Characteristics of proteolytic enzyme preparations used in fermentolysis of meat and bone raw materials.

| Indicator          | Enzyme preparation | Alcalase 2.5 L | Protamex | Protosubtilin G3x |
|-------------------|--------------------|----------------|----------|-------------------|
| Product form      |                    | thick liquid   | dark-brown | light-brown      |
|                   |                    | microgranular powder | light brown | fine powder from beige to light brown |
| Producer          | Bacillus subtilis | Bacillus complex | Bacillus | Bacillus subtilis |
| Stated activity U/g | 2.5 AU/g (Anson Units) | 1.5 AU/g (Anson Units) | Not less than 120 U/g |
| Optimum pH        | 6.5–9.0            | 5.5–7.5        | 6.5–8.5   |
| Optimum temperature, °C | 45–55              | 35–60          | 45–55     |
| Specificity       | endopeptidase      | endopeptidase  | endopeptidase |

Pre-screening was performed using Alcalase 2.5 L, Protamex and Protosubtilin G3x enzymes to identify the most effective combinations of enzymes in hydrolysis of the studied meat and bone raw materials. The EP dosage was varied in a wide range (1–3% relative to the raw material mass) with the process duration of 180 to 360 min due to high mineralization of the raw material. Fermentolysis conditions satisfied the stated optimum of all EPs (temperature 50 °C, pH 7). EP combination (endopeptidases) was prepared at equivalent dosages (0.5%, 1%, and 2% relative to the raw material mass) for 90 and 120 minutes to identify the most rational parameters of fermentolysis. Simultaneously, control experiments were performed on samples with no enzymes added.

In the experiments, 100 g of the ground raw material was placed in sealed glass jars with lids, mixed with heated water in a ratio of 1:1 by mass at 50–55 °C, and then the samples were placed in a rotary shaker for fermentolysis within a specified time. Tubular bones contain a great amount of fat (bone marrow) released during fermentolysis, therefore, it is rational to remove it by defatting the raw material to ensure the validity of the experiment. Otherwise, a stable protein-water-fat emulsion is formed, which prevents fractionation and production of pure protein fractions. To remove fat, which has high quality indicators before hydrolysis and can be considered another useful product in complex use of meat and bone raw materials, the ground raw material was mixed with hot water (heated to 80 °C), held for 30 minutes and centrifuged to separate fat.

After the end of fermentolysis, the samples were held at 90 °C for 15 minutes to inactivate enzymes. Then, the samples were centrifuged for 15 min at a frequency of 4000 rpm to separate the mixture into dense (protein-mineral) and liquid (protein) fractions.

The efficiency (depth) of fermentolysis of proteins was assessed by the content of formol-titratable nitrogen (FTN), acidity and dry matter (DM) content in the protein water-soluble fraction, and by the mass of the solid substances. Before hydrolysis, the control point was determined with respect to the FTN index. The mass fraction of water, acidity and FTN (amine nitrogen content) were determined according to GOST 7636-81.

The data obtained were processed by methods of mathematical statistics. The mathematical relationships between variable factors and quality parameters of fermentolysis were determined using the least squares method [23].

3. Results and discussion
Table 2 shows the experimental data obtained during fermentolysis of various meat and bone raw materials with individual enzyme preparations and enzyme complexes.
| Experiment number | Enzyme preparation (EP), duration | Number of EPs, % relative to the raw material mass | Characteristics of water soluble protein hydrolysate | Protein-mineral substances |
|-------------------|----------------------------------|-----------------------------------------------|--------------------------------------------------|-----------------------------|
| | | | Mass, g | Dry matter (DM), % | FTN, mg% | Titratable acidity (TA), mg% | Mass, g | Dry matter (DM), % |
| 1 | Alcalase, 180 min | 0 | 50.5 | 1.8 | 42.0 | 30.0 | 143.5 | 45.2 |
| | | 1 | 91.5 | 6.68 | 326.2 | 420.0 | 105.5 | 38.0 |
| | | 2 | 87.5 | 6.90 | 344.4 | 522.0 | 107.5 | 38.2 |
| | Alcalase, 360 min | 0 | 61.5 | 1.99 | 47.6 | 24.0 | 130.0 | 47.3 |
| | | 1 | 90.0 | 6.96 | 393.4 | 306.0 | 104.0 | 30.2 |
| | | 2 | 92 | 7.32 | 414.4 | 384 | 104.0 | 29.1 |
| 2 | Protosubtilin, 180 min | 0 | 51.0 | 1.53 | 43.4 | 24.0 | 151.6 | 49.6 |
| | | 1.0 | 74.5 | 7.67 | 331.8 | 330.0 | 124.6 | 61.3 |
| | | 2.0 | 83.1 | 8.62 | 347.2 | 468.0 | 115.9 | 62.1 |
| | | 3.0 | 87.7 | 8.89 | 337.4 | 372.0 | 113.1 | 65.1 |
| | Protosubtilin, 360 min | 0 | 58.1 | 1.88 | 51.8 | 27.0 | 140.4 | 52.5 |
| | | 1.0 | 82.6 | 6.27 | 343.0 | 234.0 | 115.4 | 63.9 |
| | | 2.0 | 90.1 | 7.8 | 370.6 | 383.0 | 110.7 | 65.6 |
| | | 3.0 | 91.1 | 8.41 | 422.2 | 393.0 | 110.7 | 66.1 |
| 3 | Protamex, 180 min | 0 | 55.0 | 1.85 | 42.0 | 24.0 | 142.0 | 52.8 |
| | | 1.0 | 88.0 | 8.00 | 249.2 | 300.0 | 109.0 | 65.4 |
| | | 2.0 | 91.0 | 9.96 | 278.6 | 480.0 | 107.0 | 65.5 |
| | | 3.0 | 93.5 | 9.39 | 246.4 | 432.0 | 105.5 | 67.4 |
| | Protamex, 360 min | 0 | 57.0 | 1.73 | 130.4 | 24.0 | 140.0 | 52.6 |
| | | 1.0 | 86.5 | 8.18 | 330.4 | 240.0 | 112.5 | 65.7 |
| | | 2.0 | 90.0 | 8.03 | 292.6 | 348.0 | 110.0 | 66.5 |
| | | 3.0 | 94.5 | 10.27 | 351.4 | 396.0 | 105.5 | 66.8 |
| 4 | Alcalase, 90 min + Protosubtilin, 90 min | 0/0 | 54.3 | 1.82 | 39.2 | 6.0 | 183.4 | 51.6 |
| | | 0.5/0.5 | 91.2 | 7.54 | 249.2 | 198.0 | 107.5 | 67.5 |
| | | 1.0/1.0 | 85.4 | 7.66 | 281.0 | 222.0 | 114.1 | 64.7 |
| | | 2.0/2.0 | 89.0 | 8.87 | 294.0 | 318.0 | 112.6 | 61.9 |
| | Alcalase, 120 min + Protosubtilin, 120 min | 0/0 | 59.0 | 2.1 | 49.0 | 6.0 | 138.4 | 51.6 |
| | | 0.5/0.5 | 85.4 | 7.83 | 267.4 | 210.0 | 118.1 | 63.8 |
| | | 1.0/1.0 | 90.6 | 8.27 | 306.6 | 306.0 | 108.9 | 65.5 |
| | | 2.0/2.0 | 84.9 | 8.50 | 260.6 | 282.0 | 117.0 | 62.7 |
| 5 | Protamex, 90 min + Protosubtilin, 90 min | 0/0 | 68.9 | 1.73 | 54.6 | 24.0 | 129.4 | 56.1 |
| | | 0.5/0.5 | 92.7 | 6.43 | 183.4 | 360.0 | 104.4 | 68.6 |
| | | 1.0/1.0 | 96.0 | 7.08 | 205.8 | 8.55 | 100.0 | 67.3 |
| | | 2.0/2.0 | 91.1 | 8.55 | 224.0 | 110.4 | 63.7 |
| | Protosubtilin, 120 min + Protosubtilin, 120 min | 0/0 | 72.6 | 1.85 | 60.2 | 18.0 | 126.8 | 55.8 |
| | | 0.5/0.5 | 99.4 | 6.47 | 212.8 | 270.0 | 99.4 | 67.6 |
| | | 1.0/1.0 | 102.0 | 7.55 | 243.6 | 408.0 | 96.7 | 68.3 |
| | | 2.0/2.0 | 96.5 | 8.22 | 245.0 | 420.0 | 103.6 | 67.8 |
The data provided in Table 2 show a complex nature of the enzymatic effect depending on the type of the raw material, EP, and fermentolysis modes. The depth of hydrolysis (indicators of FTN, DM, acidity in protein hydrolyzate) increases as the dosage of enzyme preparations grows up from 1 to 3%, while their increase is insignificant in the range of EP dosages from 2 to 3%. Masses of protein-mineral substances and the content of dry matter are hence found to decrease. This indicates conversion of collagen proteins into a soluble form under the action of enzymes, which is followed by
extraction into a water-soluble protein fraction. The best degree of hydrolysis was observed when using EP Alcalase 2.5 L at a dosage of 2% for 360 minutes and protosubtilin at a dosage of 1% for 180 minutes.

To develop mathematical models for fermentolysis suitable for assessing and forecasting this process, the graphical dependences of changes in FTN at different fermentolysis durations and EP dosages were initially analyzed in experiment 1 (Table 2, Figure 1). As can be seen, the initial rapid growth of FTN under long fermentolysis duration tends towards a certain limiting value of FTN∞, which depends on EP. Moreover, for t = 0, FTN (0, EP) = FTN0 does not depend on FTN; t \rightarrow \infty \Rightarrow FTN_{\infty}(EP). This dependence can be described by the exponential function:

\[ FTN(t, EP) = FTN_0 + (FTN_{\infty}(EP) - FTN_0) \cdot (1 - \exp(-\psi(EP) \cdot t)), \]

where FTN∞(EP) and ψ(EP) are empirical functions that should be determined based on the experimental data.

According to the experimental data, FTN0 = 19.6%. Assume FTN∞ equal to the experimental values obtained at t = 360 minutes: FTN∞ = FTN_{360}. Plot these points in Figure 2a. As is seen, the dependence of FTN∞ on EP is close to the exponential one.

In equation (1), two experimental FTN values at a given EP (initial and final) are used. There is only one experimental point (FTN_{i180}), therefore, the value of ψ can only be found from equation (1):

\[ \psi_i = -\frac{1}{180} \cdot \ln \left( 1 - \frac{FTN_{i180} - FTN_0}{FTN_{360} - FTN_0} \right). \]

The values calculated using equation (2) are plotted in Figure 2b.

Figure 1. Dependence of FTN on the duration of fermentolysis of beef ribs with EP Alcalase 2.5 L at different dosages: 1 – 0%, 2 – 1%, 3 – 2% (points are experimental data, lines are calculated using equations (1), (3), (4).

Figure 2. Empirical functions of fermentolysis of beef ribs with EP Alcalase 2.5 L: a – FTN under long duration of fermentolysis; b – indicator coefficient.
The following empirical functions can be determined using the data presented in Figure 2:

\[
FTN_{\psi}(EP) = 47.4 + 367 \cdot (1 - \exp(-1.657 \cdot EP)),
\]

\[
\psi(EP) = 0.0910 + 0.0720 \cdot (1 - \exp(-3.214 \cdot EP)).
\]

Functions (3) and (4) substituted into equation (1) can be used to calculate the lines in Figure 2, which pass through the experimental points.

The resulting regression model (equation 1) enables assessment of the main tendencies of fermentolysis, but the results should be considered evaluating since it is built using 3 points only. The model applied to the data of experiment 2 provides the dependence of FTN on the amount of protosubtilin G3x during fermentolysis of beef ribs (Figure 3).

Figure 3. Dependence of FTN during fermentolysis of beef ribs with EP protosubtilin G3x on the fermentolysis duration at different EP dosages: 1 – 0%, 2 – 1%, 3 – 2%, 4 – 3%. (points are experimental data, Table 2; lines are calculated using equations (1), (3), (4).

Similar dependencies can be constructed based on the data of experiments 3–6 to assess rational duration of fermentolysis of beef ribs in relation to FTN. For example, regardless of the dosage and type of EP, fermentolysis for more than 240–250 minutes (4 hours) is ineffective, since there is virtually no increase in FTN, and the process can cease. This can be attributed to cessation of the decomposition of raw proteins due to enzyme consumption.

The data in Table 2 show that the model employed in experiment 1 (equations 1–4) can be used for modeling the process of fermentolysis for accumulation of dry matter in the water-soluble protein hydrolyzate.

In this case, in experiment 6 performed to study fermentolysis of beef bones with proteolytic enzymes Alcalase 2.5 L, the dependence of the DM index on the fermentolysis duration can be described exponentially and take the form shown in Figure 4.

Figure 4. Change in the content of dry matter in the protein hydrolyzate during fermentolysis of beef tubular bones with EP Alcalase 2.5 L at different dosages: 1 – 0%, 2 – 1%, 3 – 2% (points are experimental data (experiment 6), lines are calculated using equations (1), (3), (4).
Similar dependences were obtained for the data of experiment 7 during fermentolysis of tubular bones with protamex enzymes (Figure 5).

![Figure 5](image5.png)

**Figure 5.** Change in the content of dry matter in the protein hydrolyzate during fermentolysis of tubular bones with EP Protamex at different dosages: 1 – 0%, 2 – 1%, 3 – 2% (points are experimental data (experiment 7, lines data calculated using equations (1), (3), (4).

When the obtained dependencies are applied to the data of experiment 5 calculated for the DM index in protein hydrolysates during fermentolysis of beef ribs with a complex of enzymes, the calculation results agree well with the experimental data (Table 2, Figure 6). When modeling FTN changes for the protein hydrolyzate in this experiment (Figure 7), the calculated and experimental data showed somewhat worse results. Apparently, 120-minute fermentolysis (Figure 7) is insufficient for maximum protein decomposition. The calculation hence yields inaccurate results since the method implies that at the last time point the limit value of the indicator is reached (or, at least, close to it).

![Figure 6](image6.png)

**Figure 6.** Dependences of changes in the content of DM in protein hydrolyzate during fermentolysis of beef ribs with a complex of enzymes (protamex + protosubtilin) at different dosages: 1 – (0% + 0%), 2 – (0.5% + 0.5%), 3 – (1% + 1%), 4 – (2% + 2%) (points are experimental data, lines are calculated using equations (1), (3), (4).
Figure 7. Dependences of changes in the FTN index in the water-soluble protein hydrolyzate during fermentolysis of beef ribs with a complex of enzymes (protamex + protosubtilin) at different dosages: 1 – (0% + 0%), 2 – (0.5% + 0.5%), 3 – (1% + 1%), 4 – (2% + 2%) (points are experimental data, lines are calculated using equations (1), (3), (4).

The inaccuracies in Figure 7 can be attributed to the fact that the data compared are obtained in fermentolysis of bones with different enzymes. Table 2 shows that the FTN values of 326.2–344.4 mg% (experiment 1 with alkalase for 180 min) and 393.4–414.4 mg% (the same experiment for 360 min) show the maximum hydrolysis of proteins of this meat and bone raw material. In experiment 5 with a complex of enzymes (protamex and protosubtilin), the depth of hydrolysis exhibits significantly lower FTN values (205–230 mg%). Thus, in experiment 5, the regression model featured the 120-minute duration as insufficient for fermentolysis.

Model (1) remains valid when the characteristic of fermentolysis decreases gradually and tends to the limit value. Thus, the model shows the change in the mass of the protein-mineral substances (PMS) in experiment 2 (Table 2, Figure 8).

Figure 8. Dependence of the PMS mass on the duration of fermentolysis of beef ribs at different dosages of EP protosubtilin: 1 – 2%, 2 – 3% (points are experimental data, lines are calculated using equations (1), (3), (4).

The obtained model and data in Figure 8 show that the process of intensive precipitation of protein-mineral insoluble substances during fermentolysis of beef ribs with protosubtilin stops after 240–250 minutes.

It is more difficult to model the parameters of fermentolysis, which behave non-monotonically in time. This is characteristic of the titratable acidity (TA) of protein hydrolysate since its values are complex since they depend on many factors (Table 2). Thus, an increase in TA at the first stage of fermentolysis (up to 180–200 min) can be explained by the occurrence of free dicarboxylic amino acids (glutamic, aspartic), which are quite abundant in this raw material and are released during protein decomposition. A subsequent insignificant decrease in the TA index is due to the occurrence of
diaminomonocarboxylic and hydroxyamino acids during hydrolysis, which prevail in the collagen proteins of raw materials (arginine, proline, histidine, glycine) that exhibit alkaline properties.

Figure 9 shows the change of EP in time in experiment 2 during fermentolysis of bones with protosubtilin (Table 2).

When modeling the dependence of TA on the fermentolysis duration, the following should be taken into account. The TA index grows up to the maximum $T_{A_m}$ value at moment $t_m$, and then it gradually decreases and tends to the steady-state $T_{A_\infty}$ value. Such changes are characteristic of overshoot processes; they can be described by the algebraic sum of exponential functions:

$$T_A(t) = T_{A_\infty} + a \cdot \exp(-\alpha \cdot t) - b \cdot \exp(-\beta \cdot t).$$  

Equation (5) contains five empirical constants: $T_{A_\infty}$, $a$, $b$, $\alpha$, $\beta$. Even approximate values of these constants cannot be obtained from three experimental points. At least six time measurements are required to build a regression model of form (5).

![Figure 9. Change of titratable acidity in time during fermentolysis of bones with protosubtilin at different dosages: 1 – 0%; 2 – 1%; 3 – 2%.](image)

4. Conclusions

Secondary meat and bone raw materials are a source of valuable organic bioactive substances (proteins, fats, protein-mineral compositions), which should be extracted using gentle hydrolytic methods.

Proteolytic enzymes used for processing of meat and bone raw materials (fermentolysis) make it possible to obtain organic fractions, primarily protein ones, which can be used in food, feed, cosmetics and other areas.

The obtained regression model of fermentolysis (equation 1) is used to determine its rational duration since longer duration is impractical as the indicators of the protein degradation depth change insignificantly.

The rational duration of fermentolysis of beef ribs and tibiae with enzyme preparations Alcalase 2.5 L, Protamex, Protosubtilin G3x is 240–250 minutes at dosages of 2–3% of the mass of the raw material.

The regression model enables forecasting the depth of fermentolysis in terms of FTN and DM content depending on the type of the raw material, enzyme preparation and fermentolysis duration.

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