SCREENING OF ASPERGILLUS FUNGI FOR EXTRA CELLULAR PROTEASE AND COLLAGENASE PRODUCTION

Abstract. Protease and collagenase are the most important enzymes used for the processing of meat raw materials. In the meat industry, proteolytic enzymes are used to accelerate the maturation of meat and increase its yield. The use of enzyme preparations in meat processing makes it possible to rationally use meat raw materials, intensify technological processes, improve quality and expand the range of products. Collagenase, unlike protease, acts on those connective proteins of meat raw materials that determine its stiffness, breaking down hard-hydrolyzable and non-digestible collagen. The aim of this study was selection of strains of industrially valuable micromycetes from the collection of micromycetes that have the ability to synthesize extracellular protease and collagenase and create a fungal association. A comparative characterization of 7 strains of micromycetes of the genus Aspergillus and Penicillium - potential producers of protease and collagenase enzymes, was carried out. A. awamori 16 and A. awamori 22 showed the highest clearance zones and was used for further studies. The clearance zones of casein of A. awamori 16 on day 5 were 22.8 mm, and collagen 20.8 mm, while the clearance zones of casein of A. awamori 22 were 20.1 mm, and collagen - 19.1 mm.

Keywords: Aspergillus, enzymes, protease, collagenase.

Introduction. Nowadays, the meat processing industry is developing new recipes and technologies using secondary meat and other food raw materials containing a sufficient amount of proteins, fats, vitamins and trace elements. In this regard, it is of great interest to use enzymes that allow the rational use of protein resources, increase the biological value of meat dishes by increasing the proportion of collagen proteolysis products – the fibrillar protein that forms the basis of connective tissue [1-3]. The use of enzyme preparations positively affects the tenderness, juiciness, nutritional value of meat raw materials, the formation of the required level of water-binding and adhesive ability, improves its organoleptic characteristics due to the targeted effect of enzymatic complexes on the components of muscle tissue [4-6].

The use of enzyme preparations in the production of meat products makes it possible to rationally use raw meat, to intensify technological processes, improve quality and expand the range of products. Of greatest interest for the processing of raw meat are the enzymes protease and collagenase. Recently, a search for microorganisms capable of intensive synthesis of these enzymes has been actively conducted. The producers of these enzymes were found among Actinomycetesrismosus, Streptomycyes griseus, Actinomycetesfradiae, etc. [7-10]. The proteolytic enzymes of bacteria of the genus Bacillus were studied. [11, 12]. Despite the fact that among microorganisms producing protease and collagenase bacteria, fungi, yeast and actinomycetes are noted, microorganisms have recently become widespread due to the ease of their cultivation and high productivity. The preparations from micromycetes of the genus Aspergillus, Penicillium, and others are successfully used [13-15].

In this regard, the selection of active strains of micromycetes – producers of enzymes and the creation based on an associative culture that will have both protease and collagenase activity.

Materials and Methods. The objects of research were micromycetes of the genus Aspergillus and Penicillium from own collection of microorganisms. The research work was conducted using accepted microbiological and biochemical research methods. The initial cultures were grown on potato – dextrose
agar for 5 days at a temperature of 30 °C. The primary selection of the culture according to the level of protease formation was carried out by a qualitative method by measuring the diameter of the clarification (hydrolysis) zones of the substrate by the cultures under study for 3-5 days of incubation (in mm) at 30 °C. Skim milk with agar was used as a substrate [16].

The primary selection of producers of collagen-cleaving enzymes was carried out in Petri dishes on Chapek-Doks medium containing purified collagen as a substrate [17]. The ability of the culture to hydrolyze the substrate was evaluated by the size of the zones of substrate hydrolysis on the 5th day of growth.

Proteolytic activity (PA) was determined according to GOST 20264.2-88 [18]. The amount of enzyme that catalyzes the hydrolysis of 1 g of protein in 30 minutes under standard conditions to products not precipitated with trichloroacetic acid was taken as a unit of proteolytic activity.

Collagenase activity was determined in the culture fluid filtrates using the method based on spectrophotometric determination of free amino acids formed during collagen hydrolysis using the ninhydrin reagent [19]. A collagen suspension was obtained by incubating the substrate in a buffer solution at 37 °C for 1 day. A buffer was prepared at pH 7.4, which contained Na2HPO4 (1.76 g/L), NaCl (8.8 g/L) in 1L of distilled water in the presence of 0.2 μM CaCl2. In order to determine collagenase activity, 1 ml of a collagen suspension was poured into a 1 ml experimental sample and the mixture was incubated at 37 °C for 18 h, after which 1 ml was taken from the incubation mixture and 2 ml of ninhydrin reagent (fresh prepared 2% ninhydrin in acetone) were added to it. It was held for 20 minutes at 100 °C, the volume of each sample was adjusted to 10 ml with distilled water, and the optical density was measured on a spectrophotometer at 600 nm.

Results and Discussion. A search for protease and collagenase producers was carried out among microscopic fungi of the genus *Aspergillus* and *Penicillium*, known as potential producers of the studied enzymes. For this purpose, a comparative characterization of 7 strains from our own collection of microorganisms was carried out – *Aspergillus awamori* 16, *Aspergillus awamori* 22, *Aspergillus awamori* 21/96, *Aspergillus oryzae* 3-9-15, *Aspergillus niger* P, *Aspergillus foetidus* and *Penicillium chrysogenum* 241. The substrate was evaluated by the size of the zones of enlightenment on the 5th day of growth (picture).

![Casein clearing zone assay](image)

The clear zone formation concerns the ability of colonies with confirmed casein hydrolysis, i.e. with the ability to synthesize an enzyme. The larger the hydrolysis zone, the more actively the culture forms an enzyme. The data obtained are presented in table 1.

According to the Table 1, the strains *A. awamori* 16 and *A. awamori* 22 were the most active in the ability to split casein and collagen. The hydrolysis zones of casein *A. awamori* 16 for 5 days were 22.8 mm, and collagen 20.8 mm, while the hydrolysis zones of casein *A. awamori* 22 amounted to 20.1 mm, and collagen – 19.1 mm. The strains of *A. oryzae* 3-9-15 and *A. niger* P, which did not produce substrate cleavage zones, had the least enzymatic activity. In order to determine the activity of protease and collagenase by selected cultures of *A. awamori* 16 and *A. awamori* 22, they were cultured under submerged conditions on a liquid nutrient medium. After 3 days, the activity of extracellular protease and collagenase was determined (table 2).
Table 1 – Selection of the active variant – producer of protease and collagenase

<table>
<thead>
<tr>
<th>Culture</th>
<th>Diameter of Casein cleavage zones (mm)</th>
<th>Diameter of Collagen cleavage zones (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus awamori 16</td>
<td>22.8±1.7</td>
<td>20.8±2.0</td>
</tr>
<tr>
<td>Aspergillus awamori 22</td>
<td>20.1±1.3</td>
<td>19.1±1.5</td>
</tr>
<tr>
<td>Aspergillus awamori 21/96</td>
<td>16.3±2.0</td>
<td>13.3±1.9</td>
</tr>
<tr>
<td>Aspergillus oryzae 3-9-15</td>
<td>0</td>
<td>16.9±1.9</td>
</tr>
<tr>
<td>Aspergillus niger rII</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aspergillus foetidus</td>
<td>11.5±1.4</td>
<td>11.3±1.2</td>
</tr>
<tr>
<td>Penicillium chrysogenum 241</td>
<td>11.2±1.7</td>
<td>12.8±1.8</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2 – Enzymatic activity of monocultures and fungal association

<table>
<thead>
<tr>
<th>Culture</th>
<th>Protease Activity, U/ml</th>
<th>Collagenase activity, U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. awamori 16</td>
<td>3.4±0.5</td>
<td>4.6±0.8</td>
</tr>
<tr>
<td>A. awamori 22</td>
<td>3.0±0.6</td>
<td>4.3±0.7</td>
</tr>
<tr>
<td>Association A. awamori 16 and A. awamori 22</td>
<td>4.2±0.6</td>
<td>6.8±0.8</td>
</tr>
</tbody>
</table>

The next stage of the research was the creation of an association of selected strains of A. awamori 16 and A. awamori 22 – producers of proteolytic and collagen-cleaving enzymes. For this purpose, a joint cultivation of selected producers in a liquid nutrient medium was carried out in deep growth conditions. After 3 days, the activity of extracellular protease and collagenase associative culture was determined. The data obtained are presented in table 2.

According to the data presented in Table 2, the association of micromycetes, consisting of A. awamori 16 and A. awamori 22 forms proteolytic and collagen degrading enzymes more actively than their monocultures. Thus, the resulting associative culture is the starting point for its further study in order to obtain an active enzyme preparation for the meat processing industry.

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организованные в чашках Петри на агаризованной среде Чапека-Докса, содержащей в качестве субстрата очищенный коллаген. Наибольшей активностью обладали штаммы A. awamori 16 и A. awamori 22. Зоны гидролиза казеина A. awamori 16 на 5 сутки составили 22,8 мм, а коллагена - 20,8 мм, тогда как зоны гидролиза казеина A. awamori 22 составили 20,1 мм, а коллагена - 19,1 мм. Следующим этапом проводимых исследований явилось создание ассоциаций из отобранных продуцентов коллагенрасщепляющих ферментов. Для этой цели было проведено совместное культивирование отобранных продуцентов в жидкой питательной среде в глубинных условиях роста. Установлено, что ассоциация микромицетов, состоящая из A. awamori 16 и A. awamori 22, активнее образует протеолитические и коллагенрасщепляющие ферменты, чем

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составляющие ее монокультуры. Так, активность протеазы *A. awamori* 16 на 3 сутки роста составила 3,4 ед./мл, а коллагеназы – 4,6 ед./мл, тогда как активность коллагеназы *A. awamori* 22 на 3 сутки роста составила 3,0 ед./мл, и коллагеназы – 4,3 ед./мл. Активность протеазы ассоциативной культуры *A. awamori* 16 и *A. awamori* 22 на третий сутки культивирования составила 4,2 ед./мл, а активность коллагеназы – 6,8 ед./мл. Дана макро- и микроморфология исходных культур и полученной ассоциации. Установлено, что на 3 сутки роста культура образует крупные колонии коричневого цвета, радиально складчатые с белым ободком. Конидиоспоры стройные, толстые, имеют гладкую поверхность. Верхняя часть конидиоспоры вдалягает и образует головки. Стеригмы представляют собой короткие цилиндрические клетки.

Ключевые слова: *Aspergillus*, ферменты, протеаза, коллагеназа.

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