RECOMBINANT M9 PEPTIDASE AS A PROMISING RAW MEAT SOFTENER

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I. INTRODUCTION

Methylotrophic yeast, *Pichia pastoris*, became the main tool in food biotechnology for the production of recombinant enzymes. A number of proteases have been successfully expressed in *Pichia pastoris*. Recent scientific papers reported about the production of recombinant buffalo chymosin used in mozzarella cheese manufacture. For meat industry, in particular for softening of meat raw material, extracellular aspartate protease was obtained from *Rhizomucor miehei* expressed in *Pichia pastoris*, whose proteolytic activity was 3480.4 U/mL [1, 2, 3]. The aim of the work was to create a food-grade genetic construct based on *Pichia pastoris* with a high-level expression of the foreign gene, namely *Aeromonas salmonicida* M9 peptidase.

II. MATERIALS AND METHODS

The objects of the study were M9 peptidase gene (ASA_3723) of *Aeromonas salmonicida* (strain of the laboratory collection isolated from the surface of meat raw material), pPic9K vector plasmid (Invitrogen, USA), competent cells of *E. coli* DH5α (Invitrogen, USA), competent cells of *Pichia pastoris* GS115 (Invitrogen, USA), culture liquid (CL) from recombinant *Pichia pastoris* clones, and samples of meat raw material (beef).
Analysis of the nucleotide sequence encoding *Aeromonas salmonicida* peptidase gene was performed using NCBI database (https://www.ncbi.nlm.nih.gov).
To analyse and compare nucleotide sequences, BLAST software was used; the search for homologous sequences was performed in GenBank database (http://www.ncbi.nlm.nih.gov/).
Bioinformation analysis of coding gene sequences for *Aeromonas salmonicida* peptidases and primer design were carried out using OligoAnalyzer Tool software (https://eu.idtdna.com/calc/analyzer).

Isolation of plasmid DNA
To isolate plasmid DNA, QIAprepSpin miniprep plasmid isolation kit (Qiagen, Germany) was used. DNA was isolated according to the manufacturer's guidelines.

PCR amplification of DNA
PCR was performed on ANK-32 instrument (Synthol, Russia) in a reaction mixture containing HS-Fuzz buffer, dNTP, 5'- and 3'-terminal primers, DNA, and HS-Fuzz DNA polymerase (NEB, England).

Preparative isolation of the DNA fragment
To isolate and purify the targeted PCR products, Cleanup Standart kit for DNA isolation from the agarose gel and the reaction mixtures (Eurogen, Russia) was used. The isolation was performed according to the manufacturer's guidelines.

Ligation-independent cloning
Cloning of the resulting PCR fragments into pPic9K vector plasmid was carried out using ligation-independent cloning system, In-FusionTM CF Dry-Down PCR Cloning Kit (Clontech Laboratories Inc., USA).

Analysis of recombinant clones for the production of target proteins by electrophoresis in PAGE
Evaluation of the enzymatic activity of CL from clones of *Pichia pastoris* was carried out by histological method. The test samples were treated with a culture liquid by injection (Experiment No. 1) and immersion (Experiment No. 2) for 24 h at 8 °C. The control meat sample was treated with saline. Histological specimens were studied using AxioImaiger A1 light microscope (Carl Zeiss, Germany) with AxioVision 4.7.1.0 digital image analysis system.

III. RESULTS AND DISCUSSION

When cloning the M9 peptidase gene into foreign cell, the absence of the target protein precursor may lead to over-synthesis of the toxic product of the foreign gene and have a negative effect on microbial cell growth and splitting. Along with expressed protein toxicity, it may lead to plasmid instability or death of the microbial host cell. To minimize this effect, a vector was used, which includes elements that reduce the expression of basal T7 RNA polymerase.
Cloning of M9 peptidase full-gene of *Aeromonas salmonicida* into pPic9K vector was carried out.
The results of assessment of the connective tissue degradation in beef shank after processing the samples with culture liquid from the recombinant *Pichia pastoris* clones with the insertion of M9 peptidase full-gene are shown in Figures 1, 2, and 3.

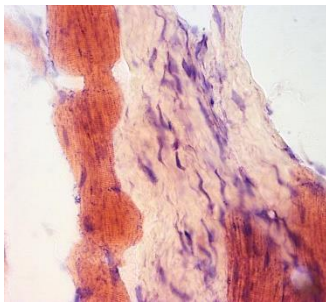

Fig. 1 Microstructure of the control sample (40x)

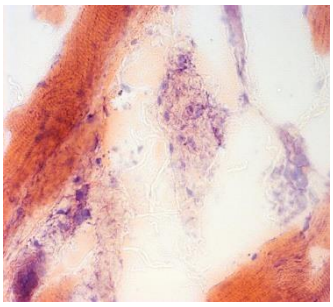

Fig. 2 Microstructure of the sample. Experiment No. 1 (40x)

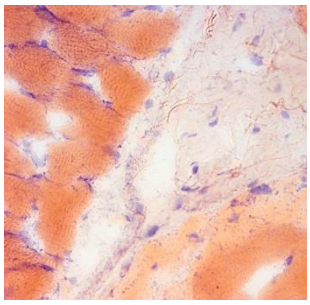

Fig. 3 Microstructure of the sample. Experiment No. 2 (40x)

In the control sample, the muscle fibers were characterized by a straightened shape, well-defined transverse striation and a fairly dense arrangement in a bundle. Oval-shaped nuclei were located directly under the fiber sarcolemma. Corrugated connective tissue layers tightly adhered to bundles of muscle fiber. The nuclei in the connective tissue layers were clearly visible in specimens (Fig. 9).
In the sample from the Experiment No. 1 (Fig. 10), changes in tissue structure were predominantly detected in deep areas in the sites of direct preparation injection. The detachment of perimysium from muscle bundles and its loosening were noted. Further, the disintegration of collagen fibrils, their thinning and partial fragmentation were revealed.
Changes in tissue structure of the sample from the Experiment No. 2, compared to the control, were similar to those in sample No. 1, but they were more pronounced on the surface, where some homogenization of the fibrillar structures was noted (Fig. 10). In deep areas, mainly, loosening of the connective tissue layers was observed.

IV. CONCLUSIONS

Cloning of M9 peptidase gene of *Aeromonas salmonicida* into pPic9K shuttle vector and transformation of *Pichia pastoris* were carried out. The samples of culture liquid from the recombinant clones were obtained.
As a result of histological studies, the effect of the resulting culture liquid from the recombinant clones on connective tissue structure of meat raw material was established. This property of the transformant, along with its safety, may be used for the registration of the obtained enzyme in food industry as a softener of meat raw material with a high collagen content.

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