CRISPR/Cas9-REDACTED *BACILLUS SUBTILIS* STRAIN AS THE PRODUCER OF EXTRACELLULAR METALLOPROTEINASE OF *B. PUMILUS*

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Abstract. *B. pumilus* metalloproteinase was firstly isolated and characterized by Kazan Federal University scientists. Primary structure analysis showed that the novel enzyme has no analogs among prokaryotic enzymes and occupies an intermediate position between two large families of the metzinkin clan metalloproteinases – adamalysins and astacins. These families are mainly represented by eukaryotic enzymes, which play an important role in human life and health. A more detailed study of the structure and functions of novel metalloproteinase requires an efficient expression system. *B. pumilus* metalloproteinase gene (*mprBp*) was cloned into the pGP382 expression vector under a strong constitutive promoter of the *degQ36* gene (P_{degQ36}). The resulting construct was used to transform *B. subtilis* Δ6 strain. This strain was constructed by CRISPR/Cas9 genome editing technology with deletion of some prophage genes of *B. subtilis* 168. The functional role of prophage genes is poorly understood. It is possible that prophage deletion will increase the expression of secreted enzymes. For the transformed strain we determined the dynamics of growth and accumulation of proteolytic activity by hydrolysis of azocasein. The dynamics of proteolytic activity accumulation by this strain has a different character in contrast to the protease-deficient strains carrying the gene of the investigated enzyme. The result of this work was to obtain an effective producer strain of adamalizin-like metalloproteinase of *B. pumilus*, which can be used in the production of the enzyme for subsequent studies.

Keywords: CRISPR/Cas9, metalloproteinase, astacins, ADAMs, *Bacillus subtilis*, *Bacillus pumilus*.

Introduction

CRISPR – clustered regularly interspaced short palindromic repeats

MprBp – extracellular metalloproteinase of *Bacillus pumilus*

Introduction

Scientists of Kazan Federal University discovered that the natural strain *B. pumilus* 3-19 secretes into the environment a minor proteinase with unknown functions and classified as zinc-dependent metalloproteinase. Analysis of the primary structure showed that the new extracellular proteinase of bacilli has no analogues among prokaryotic enzymes and occupies an intermediate classification position between two large families of metalloproteinases of the metzinkin clan – adamalysins and astacins (Rudakova *et al*., 2010, Sabirova *et al*., 2010). These families are mainly represented by eukaryotic enzymes and play an important role in human life and health. Human adamalysins are involved in epithelial tissue development, proliferation and migration of vascular smooth muscle cells, angiogenesis, apoptosis of vascular cells, tissue repair, and wound healing. Hyperproduction of these proteins can lead to diseases such as atherosclerosis, hypertension, aneurysm, coronary heart disease, myocardial infarction, and heart insufficiency (Zhong & Khalil, 2019; Malemud, 2019). Enzymes of the astacins family (mainly meprins and bone morphogenetic protein) take part in the processes of embryonic development and morphogenesis, tissue remodeling and differentiation, promote cartilage and bone tissue formation, and participate in collagen biosynthesis (Bond, 2019). All eukaryotic adamalysins are multidomain proteins. However, the catalytic domain is common to all members of the family. Structurally metalloproteinase of *B. pumilus* represents precisely the catalytic domain and it is probably evolutionary ancestor form for proteins of the adamalysins and astacins families.

To study the functions of this enzyme in the bacterial cell and to find potential applications for it, a sufficient amount of pure protein is needed. For example, it is important to obtain and characterize the protein crystal and perform X-ray analysis to characterize its three-dimen-

sional structure: features of the organization of the enzyme's active center, conformational changes upon binding to the substrate, inhibitors or cofactors, and opportunities for isomerization. This requires an efficient expression system.

Materials and Methods

Strains and plasmids

In this work, we used protease-deficient recipient strains *Bacillus subtilis* BG2036 (*ΔnprE, Δapr*) provided by prof. Eugenio Ferrarri, Genencor Int. Inc., USA, *B. subtilis* BRB08 (Δ*trpC2,* Δ*nprB,* Δ*aprE,* Δ*epr,* Δ*bpr,* Δ*nprE,* Δ*mpr,* Δ*vpr,* Δ*wprA*) and *B. subtilis* BRB14 (Δ*trpC2,* Δ*nprB,* Δ*aprE,* Δ*epr,* Δ*bpr,* Δ*nprE,* Δ*mpr,* Δ*vpr,* Δ*wprA,* Δ*htrA,* Δ*htrB*) obtained from Cobra Biologics, UK with deletion of extracellular protease genes for efficient expression of the target protein to analyze metalloproteinase gene expression. This protease-deficient strains were transformed with a genetic construct based on the plasmid vector pGP382 under a strong constitutive promoter of the *degQ36* gene (P_{degQ36}) carrying the metalloproteinase gene of *B. pumilus*.

The *B. subtilis* Δ6 strain obtained by CRISPR/Cas9 editing of the genome of the *B. subtilis* 168 strain with a deletion of the prophage genes was also chosen as a recipient (Altenbuchner, 2016). The functional role of prophage genes in bacillus genomes is poorly understood. It is possible that deletion of the prophage load will increase the expression of secreted enzymes. This means that potentially strain *B. subtilis* Δ6 can be an efficient producer of target proteins.

Nutrient media and cultivation conditions

LB agar and LB broth were used as nutrient media for cultivation *B. subtilis* strains. The antibiotic erythromycin was added to the medium at a final concentration of 10 μg / ml. For transformation *B. subtilis* Δ6 by electroporation were used the following media: SOC1 recovery medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mmol/L KCl and 10% sucrose, pH 7.0) and electroporation buffer AEB (0.5 mol/L sucrose, 1 mmol/L ammonium citrate, pH 6.0) (Shen *et al*., 2013).

Study of culture growth dynamics

Growth dynamics of recombinant *B. subtilis* strains were studied for 48, 72, 80, and 84 hours on LB medium. Bacterial growth was determined by the change in the optical density of the culture at a wavelength of λ = 600 nm and showed as OD600 on the figures.

Enzyme activity determination

The proteolytic activity of metalloproteinase *B. pumilus* was determined by the hydrolysis of azocasein (Sigma, USA) (Demidyuk *et al*., 2004) and showed as A450 on the figures.

The proportion of metalloproteinase activity in the total amount of detected proteolytic activity obtained by hydrolysis of the nonspecific substrate was determined as a ratio to the proteolytic activity level in the presence of specific metalloproteinase inhibitor 1,10-phenanthroline and showed on the figures as A450 (phen.).

Determination of culture productivity

The productivity of the culture was defined as the ratio of the value of proteolytic activity of metalloproteinase *B. pumilus* to the value of biomass and expressed in conventional units (c.u.).

DNA gel electrophoresis

DNA electrophoresis was performed in 2% agarose gel in tris-acetate buffer. A 1 Kb kit from SibEnzyme (M11, Russia) was used as markers.

Transformation of B. subtilis Δ6 cells

Transformation of *B. subtilis* Δ6 was performed by electroporation of bacterial cells according to the method (Shen *et al*., 2013) optimized by (Danilova *et al*., 2022).

To select the obtained transformants colony PCR procedure was performed.

Statistical analysis of the results

Quantitative data were summarized as means and standard deviations and compared using one-way ANOVA, followed by a Bonferroni post-hoc test to determine which groups caused the significant difference. P-values << 0.05 were considered statistically signify-

cant. All analyses were performed on 3–6 biological replicates using GraphPad Prism version 7.04.

Results

A study of the growth dynamics of *B. subtilis* Δ6 strain showed that the stationary phase of growth begins at 28-th hour of cultivation and followed by a die-off phase after 44 hours (Fig. 1). Proteolytic activity of *B. subtilis* Δ6 was detected throughout the entire growth of the culture with values not exceeding 0.24 units (Fig. 1). Also for strain *B. subtilis* Δ6 was observed a low level of culture productivity with respect to proteolytic enzyme expression.

Plasmid pGP382 (provided by T. Masher) was used as an expression vector for transformation of *B. subtilis* Δ6 carrying the gene of the metalloproteinase. The plasmid vector is characterized by the presence of a strong constitutive promoter of the degQ36 gene (P_{degQ36}). This vector contains the antibiotic resistance gene to erythromycin as a selective marker. Transformants were selected on LA medium containing erythromycin at a final concentration of 10 μg/ml. The transformation efficiency of pGP382+*mprBp* into *B. subtilis* Δ6 cells was low, and all colonies were selected for further analysis.

To confirm the presence in the transformed *B. subtilis* Δ6 strain a plasmid vector carrying *mprBp* gene, PCR amplification was performed. The selection of transformants was performed by PCR analysis and electrophoretic separation of amplified fragments in a horizontal agarose gel 2% (Fig. 2).

Protease-deficient strains were transformed with the pGP382+*mprBp* vector: *B. subtilis* BG2036 (*ΔnprE, Δapr*), *B. subtilis* BRB08 *(*Δ*trpC2,* Δ*nprB,* Δ*aprE,* Δ*epr,* Δ*bpr,* Δ*nprE,* Δ*mpr,* Δ*vpr,* Δ*wprA*) and *B. subtilis* BRB14 (Δ*trpC2,* Δ*nprB,* Δ*aprE,* Δ*epr,* Δ*bpr,* Δ*nprE,* Δ*mpr,* Δ*vpr,* Δ*wprA,* Δ*htrA,* Δ*htrB*). Study of growth dynamics of recombinant strains showed that *B. subtilis* BG2036 and *B. subtilis* BRB14 strains had similar growth curves (Fig. 3, 4).

Cultures grow exponentially up to 12–14th hour and after 30th hour there is a significant drop in the optical density of the culture. This can be explained by the transition of the population to the stage of cell death. *B. subtilis* BRB08 strain has a different growth curve. The exponential phase of biomass accumulation, just as in the previous strains, transitions to the stationary phase of culture growth by 12–14th hour. The stationary phase, not having a characteristic «plateau» pattern, decreases smoothly throughout the experiment and reaches OD600 = 0.5 by 72nd hour (Figure 5). In comparison, *B. subtilis* BG2036 and *B. subtilis* BRB14 strains reached this value of optical density by 48th hour of growth (Fig. 3 and 4). At the same time, all three strains have a maximum optical density of the culture within 1.8 (Fig. 3, 4, 5).

Significant differences between the recombinant strains are observed in the expression pattern of *mprBp*. In *B. subtilis* BG2036 and *B. subtilis* BRB08 strains enzyme is expressed in the culture fluid with a characteristic peak at the 36th and 44th hour, respectively (Fig. 3, 5). For both strains, this is the late stationary phase of growth. The maximum level of proteolytic activity for *B. subtilis* BG2036 was 4.37 units and for *B. subtilis* BRB08 was 3.37 units. Productivity of the cultures was 3.2 and 2.2, respectively (Fig. 3, 5).

For *B. subtilis* BRB14 strain the dynamics of accumulation of proteolytic activity present a different picture: the activity appears in the culture liquid at the 20th hour of culture growth, which corresponds to the stationary phase of growth, and then gradually increases in inverse proportion to the optical density of the culture. The nature of the target enzyme expression in *B. subtilis* BRB14 strain differed significantly from *B. subtilis* BG2036 and *B. subtilis* BRB08 strains. The BRB14 strain, unlike the BG2036 and BRB08 strains, was obtained by deleting all extracellular proteinases that participate in processing and perform regulatory functions in the genome. Apparently, the absence of all extracellular proteinases prevents the correct secretion and folding of the recombinant enzyme. We found that disruption of the secretome by removal of all proteolytic enzymes results in disruption of *mprBp* secretion. The maximum

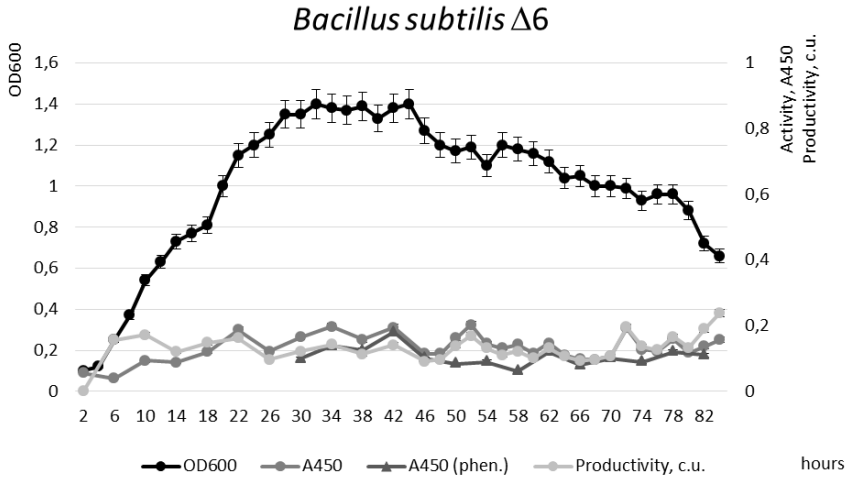

Fig. 1. Dynamics of growth and proteolytic activity accumulation of *B. subtilis* Δ6 strain

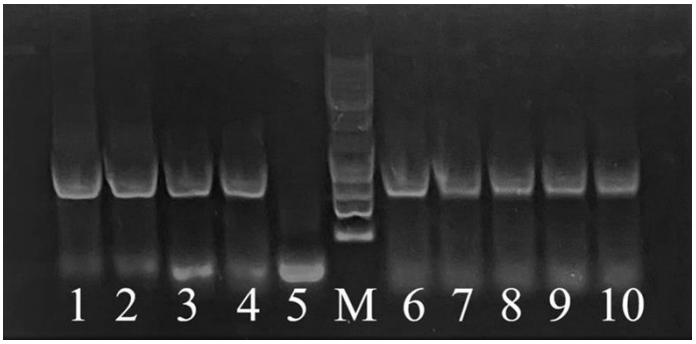

Fig. 2. Selection of transformants by colony PCR. 1 – *B. pumilus* 3-19; 2 – *B. subtilis* BG2036 (pGP382+*mprBp*); 3 – *B. subtilis* BRB08 (pGP382+*mprBp*); 4 – *B. subtilis* BRB14 (pGP382+*mprBp*); 5 – *B. subtilis* Δ6**;** M – DNA Marker (M11 1Kb, «Sibenzyme», Russia); 6-10 – *B. subtilis* Δ6 (pGP382+*mprBp*)

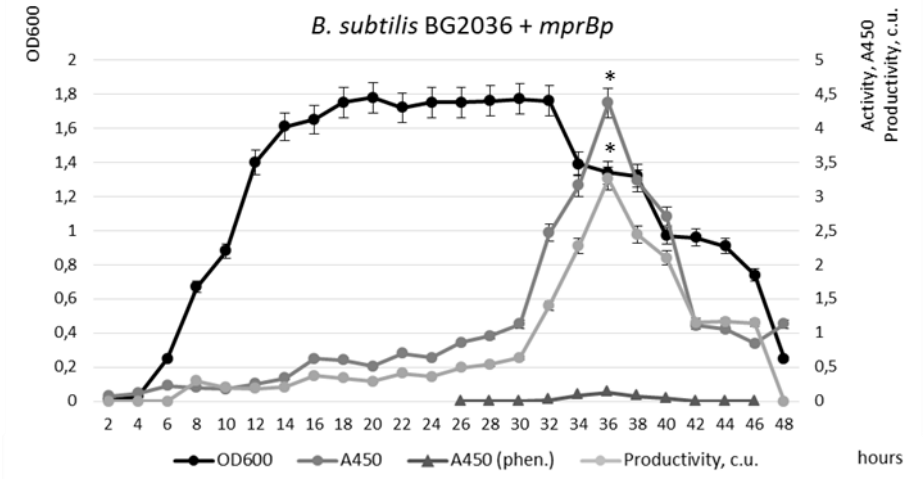

Fig. 3. Dynamics of growth and proteolytic activity accumulation of *B. subtilis* BG2036 strain

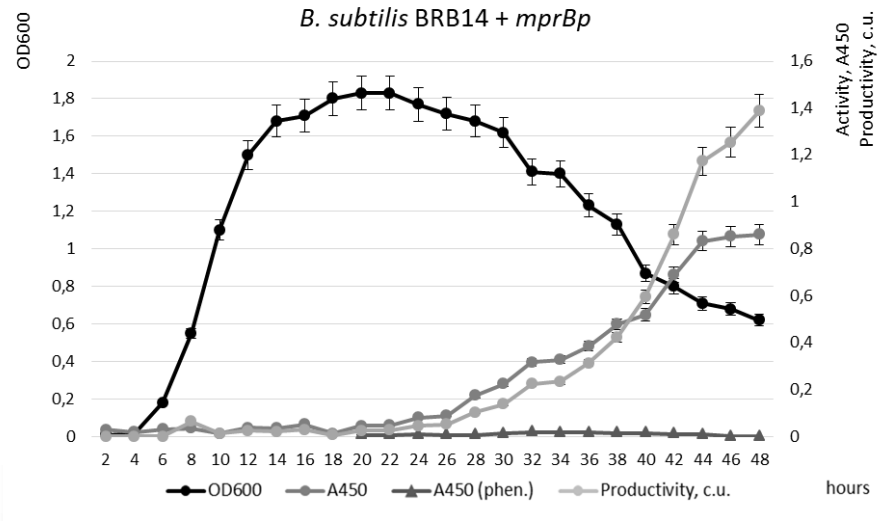

Fig. 4. Dynamics of growth and proteolytic activity accumulation of *B. subtilis* BRB14 strain

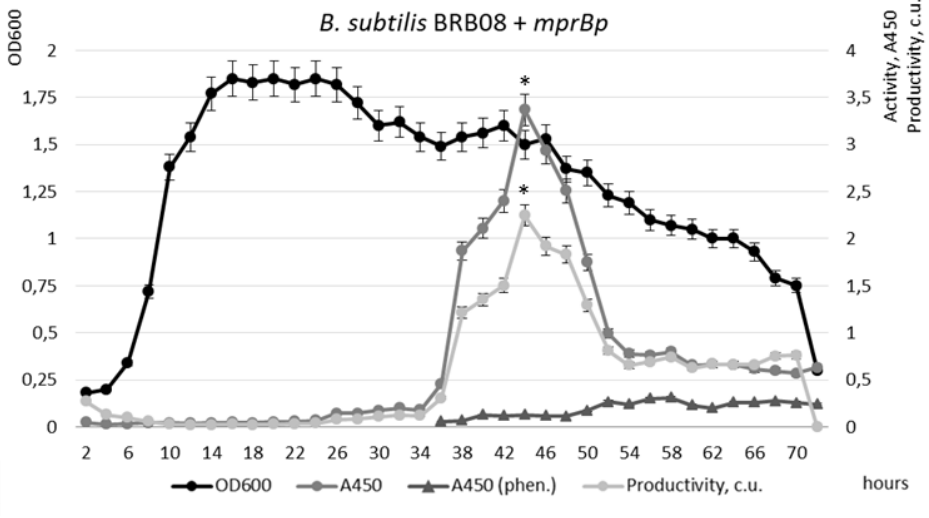

Fig. 5. Dynamics of growth and proteolytic activity accumulation of *B. subtilis* BRB08 strain

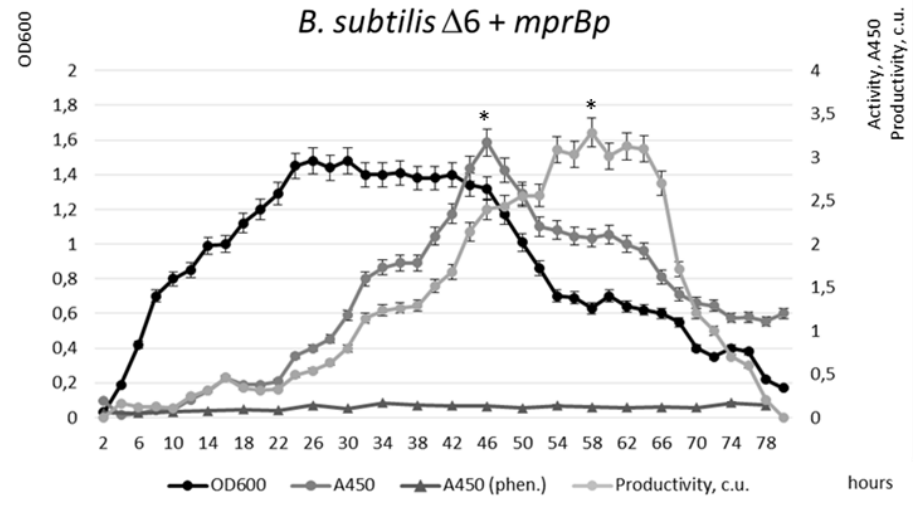

Fig. 6. Dynamics of growth and proteolytic activity accumulation of *B. subtilis* Δ6 (pGP382+*mprBp*) strain

level of metalloproteinase activity in *B. subtilis* BRB14 does not exceed 0.8 units. This is 5.5 times lower than that for *B. subtilis* BG2036 strain and 4 times lower than that for *B. subtilis* BRB08 strain. Thus, of the protease-deficient strains we used with deletions of various amounts of extracellular proteinases, the *B. subtilis* BRB14, in which all extracellular proteinases have been removed, is the least efficient producer. The probable reason for impaired MprBp secretion in the *B. subtilis* BRB14 strain is the absence of *htrA* and *htrB* proteinase genes in it. The products of these genes are the "quality control proteinases" of the Sec-dependent protein secretion pathway in bacilli. The proteinases HtrA and HtrB also promote the folding of translocated proteins (Neef *et al.*, 2020).

Because azocasein is not a specific substrate for metalloproteinases, to determine the proportion of MprBp activity in the total pool of detected proteolytic activity, we determined activity in the culture supernatant in parallel by hydrolyzing azocasein in the presence of a specific metalloproteinase inhibitor 1,10-phenanthroline. Incubation of the culture supernatant with o-phenanthroline at a concentration of 5 mM reduced the level of proteolytic activity almost to zero (Fig. 3, 4, 5). This indicates that all proteolytic activity detected in the supernatant of recombinant strains is attributable to the metalloproteinase MprBp.

The *B. subtilis* Δ6 strain due to the lack of prophage regions was interesting to us as a possible efficient producer of MprBp. The function of the deleted prophage genes is poorly understood. A study of its growth and the nature of the accumulation of proteolytic activity showed that the loss of prophage regions did not reduce an overall viability of the strain. The growth curve pattern is comparable with recombinants based on protease-deficient strains. The level of proteolytic activity in *B. subtilis* Δ6 strain does not exceed 0.24 units.

Presumably, the absence of prophage regions present in the protease-free strains may have a more effective influence on the expression of the target enzyme than the absence of its own extracellular proteases, which may be involved in the regulatory processes.

A study of the growth dynamics of the transformed strain showed that the *B. subtilis* Δ6 strain carrying the plasmid vector with the gene of the *B. pumilus* metalloproteinase grows exponentially up to 24th hour and after 46th hour there is a gradual decrease in OD600, indicating the transition of cell culture to the stage of extinction (Fig. 6).

The expression of the investigated enzyme is carried out with a sufficiently high level of proteolytic activity throughout the growth of the strain with a peak at 46th hour, which corresponds to the late stationary phase of bacterial culture growth. The maximum level of proteolytic activity for the *B. subtilis* Δ6 strain carrying the *mprBp* gene is 3.17 units. The residual proteolytic activity in the presence of the inhibitor was not exceed than 0.17 units, indicating the predominance of MprBp in the pool of extracellular proteases.

Note that after the peak of enzyme activity in the transformed strain, a drop in the optical density of cells was observed in contrast to the untransformed strain. This may be due to an increase in the metabolic load on the microbial cell due to the use of vector under the control of a strong constitutive promoter (Fig. 1, 6). A similar pattern is observed for *B. subtilis* BG2036 and BRB08 strains (Fig. 3, 5). This may indicate the general nature of the regulation of gene expression.

Discussion

The transformed *B. subtilis* Δ6 (pGP382+ +*mprBp*) strain is a promising expression system for MprBp production. Although the values of maximum proteolytic activity for the transformant are lower than those for the *B. subtilis* BG2036 and *B. subtilis* BRB08 strains, recombinant strain shows a different pattern of enzyme expression from the protease-deficient strains.

B. subtilis BG2036 and *B. subtilis* BRB08 strains are characterized by a sharp increase in proteolytic activity up to a peak followed by a sharp drop to almost zero values. This type of expression may present certain difficulties in

the production of a protein for upcoming studies. The transformant *B. subtilis* Δ6 (pGP382+ +*mprBp*) as well as the expression systems based on protease-deficient strains, shows a peak similar for the expression of *mprBp.* But in contrast to the protease-deficient strains, in *B. subtilis* Δ6 (pGP382+*mprBp*) increase of proteolytic activity begins at the early stages of cultivation, namely at 10-12th hour of growth, demonstrating a smooth increase to peak values, and then there is no less smooth decrease in activity as the culture dies out.

Conclusion

The result of the work was construction of an effective strain-producer of adamalysin-like metalloproteinase MprBp, which can be used to obtain the enzyme for subsequent studies.

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