Creation of a recombinant *Komagataella phaffii* strain, a producer of protease K from *Tritirachium album*

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Abstract. The objects of the study were recombinant clones of *Komagataella phaffii* KS1 carrying the heterologous protease K (PK-w) gene from *Tritirachium album* integrated into their genome as well as samples of recombinant protease K isolated from these clones. The aims of this work were i) to determine whether it is possible to create recombinant *K. phaffii* KS1 clones overexpressing functionally active protease K from *T. album* and ii) to analyze the enzymatic activity of the resulting recombinant enzyme. The following methods were used: computational analysis of primary structure of the protease K gene, molecular biological methods (PCR, electrophoresis of DNA in an agarose gel, electrophoresis of proteins in an SDS polyacrylamide gel under denaturing conditions, spectrophotometry, and quantitative assays of protease activity), and genetic engineering techniques (cloning and selection of genes in bacterial cells *Escherichia coli* TOP10 and in the methylotrophic yeast *K. phaffii* KS1). The gene encoding natural protease K (PK-w) was designed and optimized for expression in *K. phaffii* KS1. The protease K gene was synthesized and cloned within the plasmid pPICZα-A vector in *E. coli* TOP10 cells. The protease K gene was inserted into pPICZα-A in such a way that – at a subsequent stage of transfection into yeast cells – it was efficiently expressed under the control of the promoter and terminator of the AOX1 gene, and the product of the exogenous gene contained the signal peptide of the *Saccharomyces cerevisiae* α-factor to ensure the protein’s secretion into the culture medium. The resultant recombinant plasmid (pPICZα-A/PK-w) was transfected into *K. phaffii* KS1 cells. A recombinant *K. phaffii* KS1 clone was obtained that carried the synthetic protease K gene and ensured its effective expression and secretion into the culture medium. An approximate productivity of the yeast recombinant clones for recombinant protease K was 25 μg/mL after 4 days of cultivation. The resulting recombinant protease has a high specific proteolytic activity: ~5000 U/mg.

Key words: protease K; gene cloning; *Komagataella phaffii*; gene expression; enzymatic activity.

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Получение рекомбинантного штамма *Komagataella phaffii* – продуцента протеазы K из *Tritirachium album*

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Аннотация. Объектами исследования являлись рекомбинантные штаммы *Komagataella phaffii* K51, несущие интегрированный в их геном гетерологичный ген протеазы K (PK-w) из *Tritirachium album*, а также препарат рекомбинантной протеазы K, полученный из этих штаммов. Целью работы было изучение возможности получения рекомбинантных штаммов *K. phaffii* K51, обеспечивающих высокий уровень синтеза функционально активной протеазы K из *T. album*, и анализ ферментативной активности полученного рекомбинантного энзима. В работе использованы методы компьютерного анализа первичной структуры гена протеазы K, молекулярно-биологические методы (ПЦР, электрофорез ДНК в агарозных гелях, электрофорез белков в SDS-ПААГ в денатурирующих условиях, спектрофотометрия, методы количественного определения активности протеаз), генно-инженерные методы (методы клонирования и селекции генов в бактериальных клетках *Escherichia coli*).
Introduction

More than 70% of enzymes used in various fields of industry are hydrolases (Kudryavtseva et al., 2008; Yin et al., 2014), and proteases account for more than 30% of the total market of industrial enzymes (Kulkarni et al., 1999; Gupta et al., 2002; Koga et al., 2014; Singh et al., 2016). This is due to their widespread use in various areas of industry, in particular in the production of detergents, in waste disposal, and in the food, dairy, leather, pharmaceutical, and textile industries. The increased demand for the production of proteases in recent years is caused by the urgent need to manufacture high-quality effective detergents as well as new food products from agricultural waste of plant raw materials and from waste of meat and fish processing. In the past three decades, proteases from various sources (bacteria, bacilli and fungi) have found many applications in various fields of industry and in clinical practice. The most studied group of proteolytic enzymes is bacterial serine proteases. They are actively used in the pharmaceutical industry, in tissue engineering, and systemic enzyme therapies (Gupta et al., 2002; Kudryavtseva et al., 2008; Yin et al., 2014).

Formulations containing proteases are widely employed in many fields of medicine: in surgery – for the treatment of trophic ulcers, abscesses, phlegmons, osteomyelitis, and other purulent-inflammatory processes; in dentistry – for the treatment of caries, pulpitis, periodontitis, periodontal disease, and its complications, and in pulmonology – as a mucolytic drug for the treatment of various types of pneumonia and bronchitis (administration via inhalation).

Numerous independent studies confirm that serine proteases hold promise for medical purposes (Yarisswamy et al., 2013; Muthu et al., 2017; Belov et al., 2018; Abaturov, 2020; Osmolovsky et al., 2020). In particular, one of the main problems that physicians face when treating skin wounds and burns in people with compromised immunity is the formation of a surface biofilm generated by conditionally pathogenic microorganisms (Staphylococcus aureus and S. epidermidis, micrococci, and Pseudomonas); under this biofilm, the microbes cannot be reached by antibiotics, and as a consequence, wound healing slows down.

For the degradation of various components of the biofilm extracellular matrix, various formulations are currently being designed based on a mixture of enzymes: proteases (including protease K), glycosidases, and deoxyribonucleases (Abaturov, 2020). One of the promising areas for application of proteases is the creation of thrombolytic drugs on the basis of these enzymes. Thus, the development of new effective therapeutics based on enzymes of bacterial origin has good potential for modern medicine, microbiology, and biotechnology.

Thermostable proteases are the most popular in this regard because, firstly, they are characterized by a higher rate of catalysis, and secondly, they provide protection of the reaction mixture and products of enzymatic conversion from microbial contamination because these enzymes catalyze the reactions at high temperatures. Both bacterial and yeast strains that are recombinant superproducers of thermostable proteases have been constructed, and in most studies, it has been shown that the methylotrophic yeast Komagataella phaffii generates a larger amount of recombinant proteases than bacterial strains do (Kim et al., 2005; Latifi et al., 2013; Yu et al., 2014; Ma et al., 2016; Shu et al., 2016; Kangwa et al., 2018; Pereira et al., 2020). In addition, the proteases produced by yeasts are usually secreted into the culture medium in a soluble functionally active state (Yang et al., 2016). Of particular interest are proteases that exert their activity in a wide range of temperatures and pH of the medium.

Accordingly, a solution to the problem of obtaining a yeast superproducer of a protease from Trichiram album (proteinase K) is undoubtedly intriguing because this protease has a number of important practical advantages: it has broad specificity, is most active at high reaction temperatures (37 to 60 °C), is functional across a wide pH range (4–12), and is not inhibited by ionic or nonionic detergents. The present study is aimed at solving this problem.

Materials and methods

Materials. All chemical reagents of analytical purity were purchased from Sigma-Aldrich (USA) or Reachen (Moscow, Russia), and restriction endonucleases from SibEnzyme (Novosibirsk, Russia). DNA ligase T4 and DNA polymerase Phusion were acquired from Thermo Fisher Scientific Inc. (USA), and oligonucleotides – Biosintez (Novosibirsk, Russia). Yeast extract, bacoprotein, and tryptone from Difco were utilized to prepare the Luria-Bertani (LB) medium for growing Escherichia coli cells. Yeast culture media (YPD, BMG, BMM2, and BMM10) were prepared as described in the manufacturer’s protocol (EasySelect™ Pichia Expression Kit (Invitrogen, USA). Modified Eagle medium (MEM) was bought from Biolot (Russia), dithiothreitol and iodoacetamide,
from Bio-Rad (USA), and porcine trypsin, from Promega
(Trypsin Gold, Mass Spectrometry Grade, USA). DEAE-
Sephrose FF and SP Sepharose FF ion exchange resins were
purchased from GE Healthcare Bioscience (Sweden). The
water used in the work was deionized and autoclaved.

**Bacterial and yeast strains and plasmid vectors.** The yeast K. phaffii
K51 strain was obtained from the Russian National Collection of
Industrial Microorganisms (cat. No. Y-4935), whereas E. coli TOP10 and the pPICZα-A plasmid vector were acquired from Invitrogen Inc. (USA).

**Buffers and culture media.** Solutions and buffers were
prepared from deionized autoclaved water. E. coli clones carry-
ing the pPICZα-A plasmid or its derivatives were selected on
low-salt LB agar plates (1 % of tryptone, 0.5 % of yeast extract, 0.5 % of NaCl, 1.8 % of Bacto-agar, and 50 μg/mL zoeic). Yeast cells were grown in the YPD medium (2 % of yeast peptone, 1 % of yeast extract, and 2 % of dextrose).

Yeast transformants were cultured and selected on YPD agar plates (2 %) with various concentrations of zoeic (500 or
2000 μg/mL). The selected yeast clones were also cultivated in
the BMGY medium (1 % of yeast extract, 2 % of peptone, 100 mM potassium phosphate pH 6.0, 1.34 % of YNB, 4 × 10⁻⁵ % of biotin, and 2 % of glycerol). To induce the AOX1
promoter, the clones were cultivated first in the BMM2 medium (1.34 % of YNB, 4 × 10⁻⁵ % of biotin, and 1 % of methanol) and then in the BMM10 medium (1.34 % of YNB, 4 × 10⁻⁵ % of biotin, and 5 % of methanol).

**Construction of recombinant plasmid (pPICZα-A/ PK-w).** The nucleotide sequence of a synthetic gene encoding
natural proteinase K from T. album (i.e., protease K, endopeptidase K; E.C.3.4.21.64) – hereinafter referred to as
PK-w – was designed and optimized for expression in the yeast
K. phaffii. The optimized PK-w protease gene was synthesized by GenScript (USA). The PK-w protease gene was cloned in
E. coli cells after insertion into the pPICZα-A plasmid at the
XhoI and XbaI sites.

**Small-scale preparation of recombinant proteinase K (PK-w).** The genetically modified yeast strain was grown in
250 mL of the BMGY medium with 1 % of glycerol in 1 L
flasks on an orbital shaker at 250 rpm for 48 h at 28 °C. Next,
protein biosynthesis was induced with 1 % methanol (every
day, 25 mL of 10 % methanol was added) for 4 days. On the 4th
day, proteolytic activity in the culture liquid was determined.

**The protein concentration in solutions** was determined by three methods: a) via absorption measurement in the pro-
tein solution at 280 nm, taking into account the extinction
coefficient for the protein; b) by densitometry of the colored
protein band in a gel; c) by the Bradford assay with the Quick
Porator (Bio-Rad). The transformed cells were inoculated into
the appropriate range of tyrosine concentrations.

**Quantitation of protease activity in the culture liquid** toward azocasein. A culture liquid was centrifuged at 4 °C
for 10 min at 10000 g to pellet the cells, and the supernatant
was collected for the protease activity assay.

The reaction mixture consisting of 0.5 mL of a 0.2 % solu-
tion of azocasein in 50 mM Tris-glycine buffer (pH 8) and
0.25 mL of the supernatant was heated in a water bath at 55 °C
for 40 min. The reaction was stopped by the addition of 1 mL
of 1.2 M trichloroacetic acid. A control solution containing
0.5 mL of a 0.2 % solution of azocasein in 50 mM Tris-glycine
buffer (pH 8) without the supernatant was heated in a water
bath at 55 °C for 40 min. The reaction was stopped by the
addition of 1 mL of 1.2 M trichloroacetic acid, after which
0.25 mL of the supernatant was introduced into the mixture.
The product of the azocasein hydrolysis was quantified spe-
trophotometrically by means of absorption at 440 nm.

**Results**

**Design of the plasmid for the expression of the protease K gene in the yeast K. phaffii K51**

To obtain a yeast producer of proteinase K (i.e., protease K, endopeptidase K; E.C. 3.4.21.64), the protease gene from
T. album was optimized for expression in the methylotrophic yeast
K. phaffii K51. The amino acid sequence of the prote-

**Cloning of the recombinant plasmid pPICZα-A/PK-w in E. coli TOP10 cells**

Electrocompetent E. coli TOP10 cells were transformed with recombinant plasmids (pPICZα-A/PK-w) using an electro-
porator (Bio-Rad). The transformed cells were inoculated into
1 mL of the LB medium and incubated at 37 °C for 1 h on an
orbital shaker at 140 rpm. The cell suspensions were plated on
agar Petri dishes containing 50 μg/mL zeocin and were utilized to prepare thermolysates for colony PCR detecting the recombinant
plasmid (pPICZα-A/PK-w). The PCR was carried out with a pair of primers specific for the regions of the pPICZα-A vector
flanking the inserted gene: forward primer No. 324-AOX1-F, 5’-GAACAGTTCAATTTGACACGCT-3’; and reverse primer
No. 325-AOX1-R, 5’-GCAAATGGCATCTGGCATCC-3’.
Fig. 1. Domain structure of proteinase K (a) and amino acid sequence of the PK-w precursor protein (b).

The signal peptide is highlighted in blue, the prepeptide (prodomain) is highlighted in yellow, and the mature protein is orange.

Amplicon sizes were analyzed by electrophoresis in a 0.8 % agarose gel containing ethidium bromide. PCR-positive clones were selected for subsequent lab scale production of recombinant plasmids with the aim of their subsequent transfection into yeast cells.

Fig. 2 shows the results of testing E. coli TOP10 clones by colony PCR for the presence of the pPICZα-A plasmid carrying an insert of the proteinase K gene (named as the pPICZα-A/PK-w plasmid).

As readers can see in Fig. 2, the amplicons obtained from two recombinant plasmids containing the inserted proteinase K gene have the theoretically expected size: ~1626 bp. The selected clone containing the pPICZα-A/PK-w plasmid with the insertion of the proteinase K gene (PK-w) was used for further procedures: lab scale production of the plasmid, its linearization with restriction endonuclease BstXI, and subsequent transfection into K. phaffii K51 yeast cells.

Transfection of the proteinase K gene into K. phaffii K-51 yeast cells and screening of transfectants

At the first stage, the selected E. coli clone was propagated in 100 mL of the LB medium, followed by isolation of pPICZα-A/PK-w plasmid DNA from the cells by means of the GenElute™ HP Plasmid Midiprep Kit. The isolated plasmid DNA was analyzed by electrophoresis in a 0.8 % agarose gel stained with ethidium bromide.

DNA concentration in the plasmid sample was determined on a Qubit fluorometer (Invitrogen). As a result of the isolation procedure, ~25 µg of the purified pPICZα-A/PK-w plasmid was obtained. Approximately 5–10 µg of the isolated pPICZα-A/PK-w plasmid was linearized by digestion with restriction endonuclease BstXI and used for transfection (electroporation of K. phaffii K51, see below). At the end of the restriction reaction, phenol-chloroform extraction of DNA was carried out, followed by its precipitation with isopropanol and washing with 70 % ethanol. DNA pellets were dissolved in 10 μL of double-distilled H₂O, frozen, and stored at −20 °C. The completeness of the plasmid hydrolysis reaction was verified by electrophoresis of restriction products in a 0.8 % agarose gel stained with ethidium bromide. Judging by the results of the electrophoretic analysis, the bulk of the plasmid sample was successfully hydrolyzed by restriction endonuclease BstXI. For the transformation of electrocompetent K. phaffii K51 cells, 13–15 µg of plasmid DNA was employed, dissolved in 10 µL of double-distilled H₂O. Electroporation was performed using a Gene Pulser Xcell Total System Electroporator (Bio-Rad).

After preliminary cultivation on an orbital shaker at 200 rpm for 2 h at 27 °C in test tubes containing 1 mL of the YPD medium, the transformed cells were plated on Petri dishes with the agar YPD medium containing 500 or 2000 µg/mL zeocin. The culture plates were placed in a thermostat at 30 °C for 3–5-day incubation. On the 4th day after the cell transfection with the pPICZα-A/PK-w plasmid, many separate colonies were visible on the culture plates with 500 µg/mL of zeocin, whereas on the plates with 2000 µg/mL zeocin, there were 50 to 100 colonies. Such a large number of colonies on the plates with 2000 µg/mL zeocin is apparently due to partial degradation of the antibiotic, which had been stored at 4 °C for a long time.

Zeocin-resistant transformants grown on the culture plates with 2000 µg/mL zeocin were evaluated for their ability to synthesize and secrete the desired protein: we cultured the selected clones in 96-well deep well plates (Axygen Scientific). For screening, 20 colonies were randomly chosen, which were placed into the wells of these plates. In parallel, the same colonies, grown on agar plates with a zeocin concentration of 2000 µg/mL, were transferred to separate Petri dishes (containing an agar medium with the same zeocin concentration) by puncturing sites labeled with numbers.

The chosen clones were cultivated individually in 96-well deep well plates in 300 µL of the BMGY medium on an orbital shaker at 250 rpm for 48 h at 28 °C. Then, 250 µL of
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Fig. 3. An electropherogram of the proteins produced by the tested yeast clones transformed with the pPICZα-A/PK-w plasmid. Lanes: 1 – Thermo Scientific Molecular Weight Markers (10–200 kDa); 2–10 – proteins produced and secreted by the analyzed clones.

Fig. 4. Proteolytic activity of the culture liquid of recombinant yeast clone No. 10 as a result of its small-scale propagation in the presence of ampicillin (PK-wild/amp) or propionic acid (PK-wild/propionic acid) or without any antibacterial agents (PK-wild). The proteolytic activity of the culture liquid obtained without the antibacterial agents (PK-wild) was set to 100 %.

Fig. 5. Dependence of the recombinant proteinase K’s activity on pH and temperature.

As presented in Fig. 4, all zeocin-selected clones produce a major protein with a molecular weight of ~29–30 kDa, which corresponds to the molecular weight of mature proteinase K: 28,903 Da. According to the electrophoresis results, clone No. 10 was chosen for further experiments because it produced the largest amount of a recombinant protein with a molecular weight of ~29–30 kDa. The yield of recombinant protease PK-w after 4 days of cultivation of recombinant yeast clone No. 10 in the 96-well plate was 25 μg/mL.

Propionic acid was applied to control bacterial contamination during lab scale production of large amounts of the recombinant protein. Fig. 4 shows that the use of propionic acid at a concentration of 0.025 % is comparable to the addition of ampicillin at 0.2 mg/mL.

Microscopic analysis of the cultures did not reveal the presence of bacteria; however, the use of ampicillin and propionic acid somewhat increased the production of the target protein.

Recombinant yeast clone No. 10 was utilized for preliminary small-scale preparation of the enzyme.

Quantification of the protease activity of the recombinant protein produced by clone No. 10

As a result of these measurements, it was found that the obtained batch of the recombinant protein with a molecular weight of ~29–30 kDa had a high specific proteolytic activity, ~5000 U/mg. This finding indicated that this recombinant protein is proteinase K (PK-w).

The dependence of the obtained recombinant proteinase K’s activity on pH of the medium and on reaction temperature was investigated next (Fig. 5). The optimum of enzymatic activity
of the obtained recombinant proteinase K is in the range of pH 10–11, although the enzyme is also active at pH from 9.5 to 6.5 (see Fig. 5, a). A sharp drop of the activity was observed at pH 9.0 and at pH ≤ 4.0. The optimal temperature range for the manifestation of this protease activity turned out to be 40–65 °C (see Fig. 5, b).

Chromatographic purification of the obtained recombinant proteinase K

All procedures were conducted at a temperature ≤ 5 °C. The culture fluid of recombinant clone No. 10 was separated from the cells by centrifugation for 25 min at 4000 rpm. Low-molecular-weight impurities were removed and the supernatant was concentrated 20-fold via ultrafiltration by means of centrifugal concentrators.

Protein impurities were removed from the enzyme sample by ion exchange chromatography on anion exchange resin DEAE-Sepharose 6H. Elution was performed with a buffer composed of 50 mM sodium chloride and 50 mM Tris-HCl (pH 7.2). Fractions showing proteolytic activity were pooled, concentrated using the centrifuge concentrators, and either lyophilized or stored in 50 % glycerin in a freezer of a refrigerator.

The purified recombinant protein was analyzed by electrophoresis in SDS 12.5 % polyacrylamide gel (Fig. 6). As presented in Fig. 6, on the gel, the recombinant protein is represented by one major band with a size in the range of ~26.5–27.0 kDa. There are no protein impurities on the gel. The activity of the purified protein was 49 800 U/mg toward azocasein and 5000 U/mg toward casein. Thus, a highly purified batch of recombinant proteinase K was obtained.

Conclusion

The design and optimization of the nucleotide sequence encoding the precursor protein of natural proteinase K (PK-w) from T. album were performed to ensure its efficient expression in the yeast K. phaffii. The synthesized proteinase K gene was cloned within the pPICZα-A vector in E. coli str. TOP10 cells, and then the plasmid was isolated and transfected into yeast K. phaffii str. K51 cells.

A recombinant clone of K. phaffii K51 that carries the gene of recombinant proteinase K and successfully expresses and secretes this enzyme into the culture medium was obtained. A lab scale batch of the recombinant proteinase K was prepared. Protease activity of the obtained recombinant proteinase K (PK-w) was determined with casein and azocasein as substrates. The enzyme batch has a high specific proteolytic activity: ~5000 U/mg. The optimal enzymatic activity of the obtained recombinant proteinase K is in a pH range of 10–11 and a temperature range of 40–65 °C.

References

Abaturov A.E. Proteases that degrade the biofilm matrix. Zdorov’ye Rebenka = Child’s Health. 2020;15(3):187-194. DOI 10.22241/2224-0551.15.3.2020.204545. (in Russian)

Belov A.A., Vaniushenкова A.A., Dosadina E.E., Khanafina A.A. New textile dressings based on biodegradable polymers containing proteinases for wounds and burns treatment. Wounds and Wound Infections. The prof. B.M. Kostytchenok Journal. 2018;5(1):16-26. DOI 10.25199/2408-9613-2018-5-1-16-26. (in Russian)

Bisswanger H. Practical Enzymology. Moscow: Binom Publ., 2010; 154-156. (in Russian)

Gupta R., Beg Q., Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 2002;59(1):15-32. DOI 10.1007/s00253-002-0975-y.

Kangwa M., Salgado J.A.G., Fernandez-Lahore H.M. Identification and characterization of N-glycosylation site on a Macor circinelloides asparagine protease expressed in Pichia pastoris: effect on secretion, activity and thermo-stability. AMB Express. 2018;8(1):157. DOI 10.1186/s13568-018-0691-3.

Kim T., Lei X.G. Expression and characterization of a thermostable serine protease (TfpA) from Thermomonospora fusca XY in Pichia pastoris. Appl. Microbiol. Biotechnol. 2005;68(3):355-359. DOI 10.1007/s00253-005-0191-8.

Koga Y., Tanaka S.-I., Sakudo A., Tobiume M., Aranishi M., Hirata A., Takano K., Ikuta K., Kanaya S. Proteolysis of abnormal prion protein with a thermostable protease from Thermococcus kodakarensis KOD1. Appl. Microbiol. Biotechnol. 2014;98(5):2113-2120. DOI 10.1007/s00253-013-5091-7.

Kudryavtseva O., Dunavskys V., Kamzolinka O., Belozersky M. Fungal proteolytic enzymes: features of the extracellular proteases of xylotrophic basidiomycetes. Microbiology. 2008;77(6):643-653. DOI 10.1134/s0026261708060015.

Kulkarni N., Shendye A., Rao M. Molecular and biotechnological aspects of xylanases. FEMS Microbiol. Rev. 1999;23(4):411-456. DOI 10.1111/j.1577-6676.1999.tb00407.x.

Lattifi A.A., Salleh A.B., Rahman R.N., Oslan S.N., Basri M. Secretory expression of thermostable alkaline protease from Bacillus stearothermophilus FL by using native signal peptide and α-factor secretion signal in Pichia pastoris. Genes Genet. Syst. 2013;88(2):85-91. DOI 10.1266/ggs.88.85.

Ma X., Liu Y., Li Q., Liu L., Yi L., Ma L., Zhai C. Expression, purification and identification of a thermolysin-like protease, neutral protease I, from Aspergillus oryzae with the Pichia pastoris expression system. Protein Expr. Purif. 2016;128:52-59. DOI 10.1016/j.pep.2016.08.008.

Muthu S., Gopal V.B., Soundararajan S., Natarayan K., Narayan K.S., Lakshminikanth M., Malairaj S., Perumal P. Antibacterial serine proteases for wounds and burns treatment. Wounds and Wound Infections. The prof. B.M. Kostytchenok Journal. 2018;5(1):16-26. DOI 10.25199/2408-9613-2018-5-1-16-26. (in Russian)
protease from *Wrightia tinctoria*: Purification and characterization. *Plant Physiol. Biochem*. 2017;112:161-172. DOI 10.1016/j.plaphy.2017.01.003.

Osmolovskaya A.A., Orekhova A.V., Konti E., Kreyer V.G., Baranov N.A., Egorov N.S. Production and stability of the protease complex from *Aspergillus ochraceus* L-1 with fibrinolytic and anti-coagulant activity. *Moscow University Biological Sciences Bulletin*. 2020;75(3):130-135.

Pereira W.E.S., da Silva R.R., de Amo G.S., Ruller R., Kishi L.T., Boscolo M., Gomes E., da Silva R. A collagenolytic aspartic protease from *Thermomucor indicae-seudaticae* expressed in *Escherichia coli* and *Pichia pastoris*. *Appl. Biochem. Biotechnol*. 2020;191(3):1258-1270. DOI 10.1007/s12010-020-03292-z.

Shu M., Shen W., Yang S., Wang F., Wang Y., Ma L. High-level expression and characterization of a novel serine protease in *Pichia pastoris* by multi-copy integration. *Enzyme Microb. Technol*. 2016;92:56-66. DOI 10.1016/j.enzmicro.2016.06.007.

Singh R., Kumar M., Mittal A., Mehta P.K. Microbial enzymes: industrial progress in 21st century. *J Biotech*. 2016;6(2):174. DOI 10.1007/s13205-016-0485-8.

Yang H., Zhai C., Yu X., Li Z., Tang W., Liu Y., Ma X., Zhong X., Li G., Ma L. High-level expression of proteinase K from *Tritirachium album* limber in *Pichia pastoris* using multi-copy expression strains. *Protein Expr. Purif*. 2016;122:38-44. DOI 10.1016/j.pep.2016.02.006.

Yarishwamy M., Shivaprasad H.V., Joshi V., Nanjaraj Urs A.N., Nataraju A., Vishwanath B.S. Topical application of serine proteases from *Wrightia tinctoria* R. Br. (Apocynaceae) latex augments healing of experimentally induced excision wound in mice. *J. Ethnopharmacol*. 2013;149(1):377-383. DOI 10.1016/j.jep.2013.06.056.

Yin C., Zheng L., Chen L., Tan Q., Shang X., Ma A. Cloning, expression, and characterization of a milk-clotting aspartic protease gene (Po-Asp) from *Pleurotus ostreatus*. *Appl. Biochem. Biotechnol*. 2014;172(4):2119-2131. DOI 10.1007/s12010-013-0674-4.

Yu X., Wang X., Zhong X., Tang W., Zhai C., Chen W., Ma L. Expression of the thermostable carboxypeptidase Taq gene in *Pichia pastoris* GS115. *Sheng Wu Gong Cheng Xue Bao*. 2014;30(11):1791-1795.

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