PREPARATION OF A RECOMBINANT RIBONUCLEASE INHIBITOR IN *E. COLI* FOR USE IN mRNA SYNTHESIS *IN VITRO*

Zakharova MV[1], Zagoskin AA[1,2], Ivanov RA[2], Nagornykh MO[1,2] ✉

[1] Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia
[2] Sirius University of Science and Technology, Sirius, Sochi, Russia

Technologies underlying the production of synthetic mRNAs in vitro have significantly expanded the possibilities for research and therapeutic use of this class of molecules. The flagship application area has been the niche of mRNA vaccines, but this class of therapeutic molecules has the potential to be applied in a much broader range of situations. The process of *in vitro* production of artificial RNA molecules is based on an enzymatic synthesis reaction, one of the components of which is a ribonuclease inhibitor. This protein protects synthesized RNA from attacks by ribonucleases and prevents degradation of the molecules, which is critically important for RNA. Eukaryotic ribonuclease inhibitor synthesized as a recombinant protein in the cells of *E. coli* bacteria is the most common choice. However, the structure of this protein makes it a difficult product to make in bacteria. This study aimed to test the production of a recombinant ribonuclease inhibitor in various strains of *E. coli*, and to show the effect that helper polypeptides and cellular chaperones have on this process. Using genetic engineering approaches, we constructed plasmids, from which chimeric ribonuclease inhibitor molecules and helper polypeptides were produced. The influence of various components on solubility of the target recombinant protein was assessed with the help of densitometry, to which we have subjected products of the PAGE electrophoresis. It was determined that combinations of a vector with a strong promoter for the expression of the *RNH1* ribonuclease inhibitor gene and helper polypeptides MBP and TIG against the background of increased expression of cellular chaperones dnaK, dnaJ, grpE give the target product yield of 45 mg/l and 60 mg/l, respectively. The selected conditions allow large-scale production of this protein for further use in in vitro RNA synthesis in the context of production of medicines.

Keywords: ribonuclease inhibitor, production of recombinant proteins, chaperones, fusion polypeptides

Funding: the study was financially supported in the context of the program of Ministry of higher education and science of the Russian Federation (agreement #075-10-2021-113, unique project number RF----193021X0001).

Author contribution: Zakharov MV — selection of conditions of production of recombinant proteins, production experiments in different E. coli strains; Zagoskin AA — chromatographic clearing of recombinant proteins; Nagornykh MO — conceptualization, design of genetic constructs, article authoring; Ivanov RA — general management.

✉ **Correspondence should be addressed:** Maxim O. Nagornykh
Prospekt Nauki, 5, Pushchino, 142290, Russia; derbanner@gmail.com

Received: 16.11.2023 **Accepted:** 17.12.2023 **Published online:** 31.12.2023

DOI: 10.24075/brsmu.2023.058

ПОЛУЧЕНИЕ РЕКОМБИНАНТНОГО ИНГИБИТОРА РИБОНУКЛЕАЗ В *E. COLI* ДЛЯ ИСПОЛЬЗОВАНИЯ В СИНТЕЗЕ мРНК *IN VITRO*

М. В. Захарова[1], А. А. Загоскин[1,2], Р. А. Иванов[2], М. О. Нагорных[1,2] ✉

[1] Институт биохимии и физиологии микроорганизмов Российской академии наук, Пущино, Россия
[2] Научно-технологический университет «Сириус», Сочи, Россия

Технологии, лежащие в основе получения синтетических мРНК *in vitro*, значительно расширили возможности их исследовательского и терапевтического применения. Флагманской областью применения стала ниша мРНК-вакцин, однако этот класс терапевтических молекул может быть применим к гораздо более широкому кругу задач. В основе процесса получения искусственных молекул РНК лежит реакция ферментативного синтеза *in vitro*, одним из компонентов которой является ингибитор рибонуклеаз. Этот белок необходим для защиты синтезированных РНК от атак рибонуклеаз с целью предотвращения деградации молекул, что критично для РНК. Чаще всего используют ингибитор рибонуклеаз эукариотического происхождения, полученный в виде рекомбинантного белка в клетках бактерий *E. coli*. Однако он является сложной мишенью для наработки в бактериях, что обусловлено его структурой. Целью работы было проверить наработку рекомбинантного ингибитора рибонуклеаз в различных штаммах *E. coli*, а также показать влияние хелперных полипептидов и клеточных шаперонов на этот процесс. При помощи подходов генной инженерии были сконструированы плазмиды, с которых проводили наработку химерных молекул ингибитора рибонуклеаз и вспомогательных полипептидов. Оценку вклада различных компонентов, влияющих на растворимость целевого рекомбинантного белка, проводили в ходе денситометрического анализа результатов электрофореза в ПААГ. Определили, что комбинации вектора с сильным промотором для экспрессии гена ингибитора рибонуклеазы *RNH1* и вспомогательных полипептидов MBP и TIG на фоне повышенной экспрессии клеточных шаперонов dnaK, dnaJ, grpE дают выход целевого продукта 45 мг/л и 60 мг/л соответственно. Подобранные условия дают возможность крупномасштабных наработок этого белка для дальнейшего использования в синтезе РНК *in vitro* при производстве терапевтических препаратов.

Ключевые слова: ингибитор рибонуклеаз, продукция рекомбинантных белков, шапероны, вспомогательные полипептиды

Финансирование: работа выполнена при поддержке программы Министерства высшего образования и науки РФ (соглашение №. 075-10-2021-113, уникальный номер проекта RF----193021X0001).

Вклад авторов: М. В. Захарова — подбор условий наработки рекомбинантых белков, эксперименты по наработке в разных штаммах *E. coli*; А. А. Загоскин — хроматографическая очистка рекомбинантных белков; М. О. Нагорных — концепция работы, создание генетических конструкций, написание статьи; Р. А. Иванов — общее руководство.

✉ **Для корреспонденции:** Максим Олегович Нагорных
Проспект Науки, д. 5, г. Пущино, 142290, Россия; derbanner@gmail.com

Статья получена: 16.11.2023 **Статья принята к печати:** 17.12.2023 **Опубликована онлайн:** 31.12.2023

DOI: 10.24075/vrgmu.2023.058

Encoded by the *RNH1* gene, ribonuclease inhibitor is a protein expressed in the cytosol; it inhibits pancreatic ribonucleases, merging with them into complexes and protecting cellular RNA from premature degradation [1, 2]. The inhibiting effect stems from non-covalent binding of protein ribonuclease molecules, which leads to blocking of both RNA-binding and catalytic ribonuclease domains [3]. Ribonuclease inhibitor is a fairly conservative protein, with homology over 70% in mammals - humans, mice, horses, and pigs. It consists of 15–16 leucine-rich repeats (the exact number of repeats depends on the type of mammal), and each repeat includes 28–29 amino acids, has one alpha helix and one beta sheet. The repeats make up a U-shaped structure weighing approximately 50 kDa [4, 5]. Physico-chemical properties of ribonuclease inhibitor put it among acidic proteins, with isoelectric point in the region of 4.5. Thirty-two cysteine residues, which enable the enzyme, are another important characteristic of ribonuclease inhibitor. In native protein, cysteines are in a reduced state; they participate in the formation of a hydrophobic protein core. Oxidation of cysteine residues is accompanied by appearance of disulfide bridges between them, and conformational changes weaken the activity of ribonuclease inhibitor [6, 7].

Protein preparations of the ribonuclease inhibitor are of biotechnological value for the purposes of both research (*in vitro* RNA synthesis, *in vitro* translation, cDNA production) and mass production of medicines based on artificial RNA molecules. Therefore, since its discovery, there have been attempts to synthesize this enzyme in large quantities. Historically, the first of the said attempts sough to make the ribonuclease inhibitor from mammalian tissues [8]. As the recombinant DNA technologies matured, the focus of attention shifted to the most widely used *E. coli* bacterial system. However, recombinant ribonuclease inhibitor tends to aggregate in *E. coli*, like other proteins containing a large number of hydrophobic amino acids, and with cysteine residues in particular. Several other factors, including the high rate of translation associated with transcription in bacteria, bring accumulation of such recombinant proteins as inclusion bodies formed from aggregates of improperly folded protein molecules [9]. Therefore, early experiments designed to produce a functionally active soluble ribonuclease inhibitor in *E. coli* often ended with the entire protein accumulating as inactive aggregates, and when these were refolded *in vitro*, the resulting protein most often lost its potency, and the yield thereof was very low [10].

Various approaches have been developed to solve this problem. One of them suggests employing a vector with a weak promoter (for example, Ptrp) and a minimal medium for expression of the ribonuclease inhibitor gene. Such conditions weakened the expression of the target protein gene significantly, which allowed yielding considerable amounts of the recombinant inhibitor in a soluble form, but the overall level of the final product turned out to be significantly lower compared to expression systems where the target gene is controlled by the T7 promoter [2]. Another approach involves enriching the culture medium with DTT (dithiothreitol), thus keeping cysteines in a reduced state, which has a positive effect on the folding of many proteins prone to aggregation, including ribonuclease inhibitor [11]. This technique was further developed with cultivation under anaerobic conditions at a reduced temperature and with co-expression of the GroELS cellular chaperone, which brings the production of ribonuclease inhibitor to a fairly high level [12]. The downsides of this method are its complexity and the large number of additional components required, both complicating the technological process when the goal is to synthesize the protein at scale. Finally, another approach aims at producing the target product as a chimeric molecule with an fusion polypeptide that improves solubility of the target protein. In an earlier study, MBP fusion polypeptide was shown to positively affect production of the ribonuclease inhibitor in soluble form in *E. coli* [4].

The purpose of this work was to identify the conditions of production of the recombinant human ribonuclease inhibitor in *E. coli* that would simplify the process in the laboratories and facilities synthesizing the product at semi-industrial scale.

METHODS

Plasmid design

The genetic constructs used in this work were produced by the restriction ligase DNA cloning method. The DNA sequence of the *RNH1* gene encoding human ribonuclease inhibitor was synthesized *de novo* with codon optimization for *E. coli* (IDT, https://www.idtdna.com /). Amplification of all DNA sequences followed the manufacturer's protocol (NEB) and employed Q5 high-fidelity polymerase. Cloning of the ribonuclease inhibitor gene into the set of vectors with fusion polypeptides based on pET28a (Novagen; USA) and pSol (Lucigen; USA) was done by restriction sites NdeI and NotI, in an enzymatic reaction, with two corresponding restriction endonucleases (Sibenzyme; Russia); the procedure lasted for 1 hour at the temperature of 37 °C. Next, we did electrophoresis in agarose gel and purified DNA fragments therefrom with the help of a GeneJET Gel Extraction Kit (Thermo; USA). These fragments, corresponding to the vector part and the ribonuclease inhibitor gene, were then used in the ligation reaction with T4-DNA ligase (NEB), which lasted for 30 minutes at room temperature. Competent *E. coli* 10G cells (Lucigen; USA) were transformed with the help of electroporation (Bio-Rad; USA) as prescribed by the manufacturer. Plasmid DNA was isolated from clones containing correct inserts, with confirmed correctness by the Sanger sequencing reaction.

Production of recombinant proteins in *E. coli*

We used the following strains intended for production of recombinant proteins in *E. coli*: BL21 (DE3) (Invitrogen; USA); KRX (Promega; USA); Rosetta (DE3) pLysS (Novagen; USA); BL21 Star (DE3) (Invitrogen; USA), Origami 2 (DE3) (Novagen; USA). Using plasmids containing genes of fusion polypeptides and the ribonuclease inhibitor, we electroporated each strain and seeded the LB liquid medium in test tubes (35 ml, including selective antibiotics) with a transformation mixture (100 µl) . The incubation lasted for 12–14 hours at 37 °C, with the stirring speed of 180 rpm. Next, we transplanted the grown culture in a ratio of 1:200 into test tubes or flasks containing the required amount of the LB culture medium (10–500 ml) with selective antibiotics. The cell culture was built up to the optical density (OD) of = 0.5–0.6 in a shaker at 180 rpm at 37 °C. Once the needed OD was reached, we cooled the culture on ice for 10 minutes, added the 1 mM IPTG inductor for pET28a-based vectors or 0.2% L-rhamnose for pSol-based vectors, and incubated at different temperatures while stirring (120 rpm) for 10 hours (16 °C) or 2 hours (37 °C). Co-expressed cellular chaperone genes were induced by the introduced L-arabinose (0.5%) or tetracycline (5 ng/ml), with pre-incubation before adding the target gene expression inducers. Then, bacterial cells were deposited by centrifugation for 20 minutes at 6000 g and 4 °C.

To measure solubility of the resulting protein, we transferred the deposited cells into buffer A (50 mM Tris-HCl pH 7.5; 0.3

M NaCl; 0.005 M imidazole) and destroyed them using a Qsonica Q700 ultrasound disintegrator until the cell suspension on ice was clarified (ultrasonic pulses for 10 s, cooling — 60 s). The destroyed cells were deposited by centrifugation (15000 g), which lasted 30 minutes at 4 °C. Next, we collected samples of the supernatant containing the target protein in the soluble form, and the precipitate with aggregates was dissolved in urea (2 M) for further electrophoretic analysis. PAAG electrophoresis (10%) was used to separate protein under denaturing conditions, as prescribed by the standard methods. After staining with Coomassie blue dye and washing it off, we photographed the gels and quantified the soluble and insoluble forms of protein. Image Lab application (Bio-Rad; USA) enabled densitometric analysis of the gels.

Protein purification using affinity chromatography

After ultrasonic destruction of the biomass and centrifugation of cellular debris, we filtered the remaining supernatant through a membrane with 0.22 μm pores. The resulting preparation for chromatographic purification contained the soluble fraction of proteins in buffer A (50 mM Tris-HCl pH 7.5; 0.3 M NaCl; 0.005 M imidazole); it was applied on Nuvia IMAC Resin (Bio-Rad), a chromatographic sorbent. The proteins were eluated by chromatographic buffer B (50 mM Tris-HCl pH 7.5; 0.5 M imidazole). In a Spin-X centrifuge concentrator (pore size 10 kDa), purified preparation was concentrated from 5 to 1 ml. If necessary, we further treated it with TEV protease (following manufacturer's recommendations) overnight at 4 °C, and then repeated chromatographic purification, but after that only one fraction, that which did not bind to the sorbent and contained purified ribonuclease inhibitor, was collected. The target fraction was dialized against the storage buffer (40 mM HEPES-KOH pH 7.6, 100 mM KCl) at 4 °C. Glycerin (50%) and DTT (8 mM) were added for long-term storage at –20 °C.

RESULTS

Our first step in identification of the optimal conditions for production of soluble recombinant ribonuclease inhibitor was extension of the list of screened fusion polypeptides that increase solubility of this protein, since this approach has already been tested earlier [4]. For this purpose, we have constructed a set of twenty vectors based on two expression plasmids, pET28a (Novagen; USA) and pSol (Lucigen; USA). Two vectors contained the human ribonuclease inhibitor (*RNH1*) gene codon optimized for expression in *E. coli*, with its sequence encoding polyhistidine tag at the protein's N-terminus, which ultimately encoded the target protein molecule with a histidine tail for affine purification. The remaining plasmids encoded chimeric proteins consisting of fusion polypeptides (MBP, TIG, TSF, FH8, PpiB, YrhB, SUMO, TRX, GST) and a recombinant ribonuclease inhibitor. The induction of expression from T7 (pET28a) or rhamnose (in the case of pSol) promoter in these plasmids enables production of chimeric proteins comprised of combinations of a ribonuclease inhibitor with different fusion polypeptides carrying the polyhistidine tag on the N-terminus for affinity purification (Fig. 1). It should be noted that a sequence encoding the proteolysis site for TEV protease was inserted between the ribonuclease inhibitor gene and genes of the fusion polypeptides. Thus, after primary affinity purification, it is possible to split off the fusion polypeptide and use affinity chromatography to produce a pure ribonuclease inhibitor preparation.

All the resulting genetic constructs were tested under the typical conditions of production of recombinant proteins in *E. coli*; for the purpose, they transformed cells of BL21 (DE3) strain, and enabled the said production at 37 °C. As expected, incubation of bacterial cultures at 37 °C yielded no soluble form of the ribonuclease inhibitor but only insoluble aggregates, regardless of the fusion peptide and from what promoter the target gene's expression was initiated (data not provided). When the incubation temperature was lowered to 16 °C, variants without fusion peptides also formed inclusion bodies, but in the end, there were several chimeric proteins in the soluble form (Fig. 2). Therefore, all our further attempts at production of the recombinant ribonuclease inhibitor were conducted at lower temperature (16 °C) . In particular, we registered good results when using the following fusion polypeptides: MBP (70–75% of the target product in soluble form), TIG (about 90% of the target product in soluble form) and, to a lesser extent, PpiB (about 60% of the target product in soluble form).

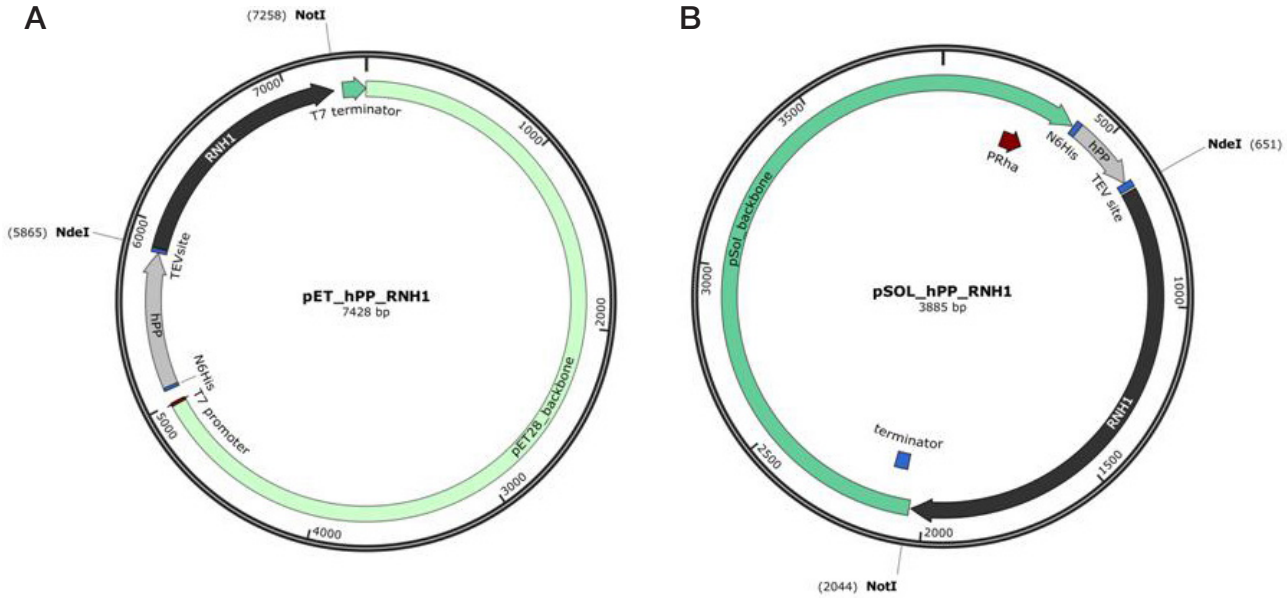

Fig. 1. Maps of vectors for production of ribonuclease inhibitor with the fusion polypeptides that increase solubility of recombinant proteins in *E. coli*. **A.** Variants based on the pET28a vector, T7 promoter. **B.** Variants based on the pSol vector, PRha promoter. *RNH1* — gene encoding the human ribonuclease inhibitor; *hPP* — gene encoding one of the fusion polypeptides (MBP, TIG, TSF, FH8, PpiB, YrhB, SUMO, TRX, GST). pET28a vector backbone contains the kanamycin resistance gene, pBR322 replication origin, and the sequence encoding the lacI repressor. pSol vector backbone contains the kanamycin resistance gene, pUC replication origin, the rhamnose-induced PRha promoter, and the transcription terminator

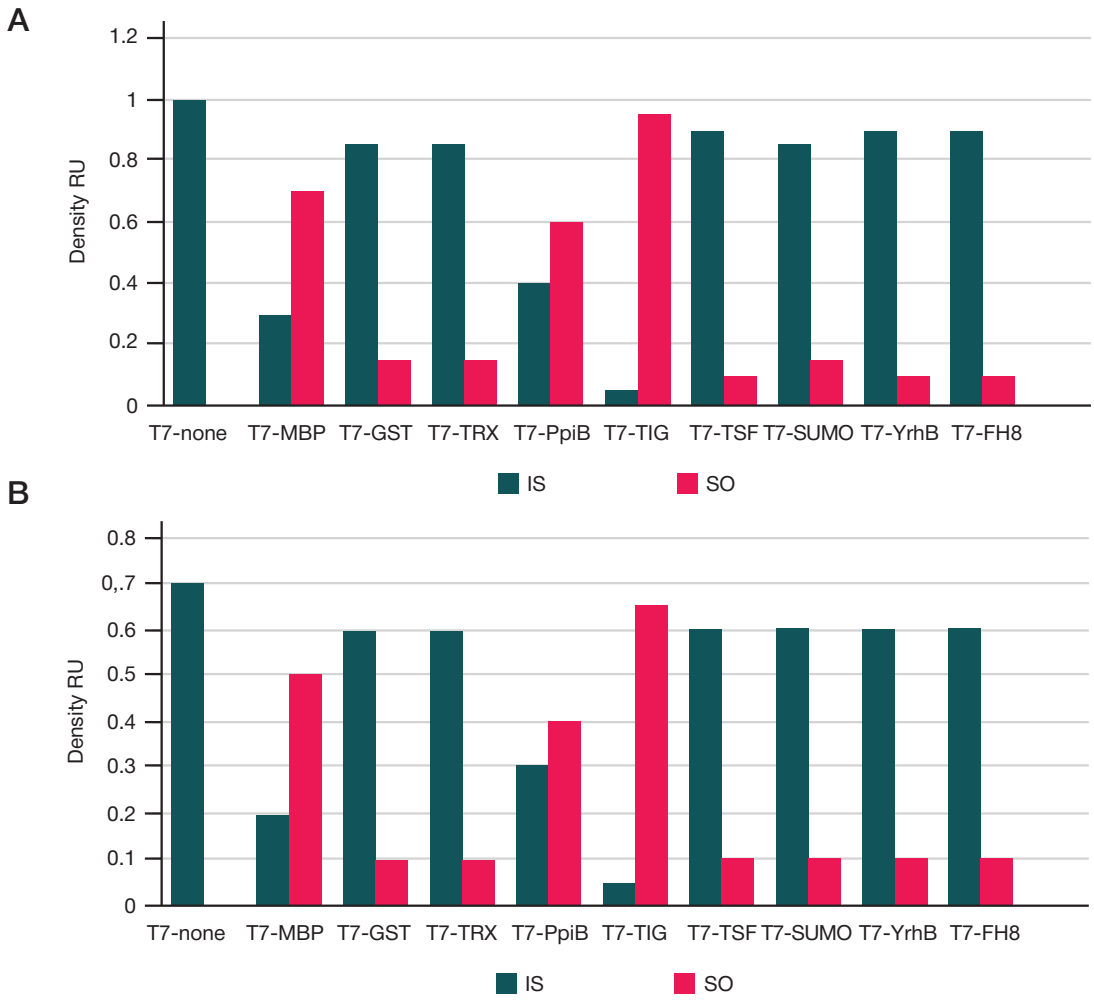

Fig. 2. Effect of fusion polypeptides on solubility of ribonuclease inhibitor, production in *E. coli* BL21 (DE3) strain at 16 °C. **A.** Variants based on the pET28a vector, T7 promoter. **B.** Variants based on the pSol vector, PRha promoter. IS is the relative level of insoluble fraction, shown in red. SO is the the level produced soluble ribonuclease inhibitor, shown in blue. Density RU are densitometry of PAAG results shown in relative units

Stronger promoter T7 ensured considerably bigger yield of the protein in pET28a vector systems compared to the pSol vector, which relies on a weaker rhamnose promoter (Fig. 2). Nevertheless, part of the target protein still transitioned into a functionally inactive state in the inclusion bodies, a problem that could be remedied by further optimization of the operating conditions.

The next step was to compare the yield of soluble ribonuclease inhibitor in different *E. coli* strains designed specifically for the production of recombinant proteins. As a rule, previous attempts on the task incorporated the experience of working with recombinant producers based on the *E. coli* BL21 (DE3) strain, but, in most cases, it is not the optimal choice in case of proteins prone to aggregation: a significant proportion of the product are insoluble aggregates. Therefore, seeking to refine the conditions of production of ribonuclease inhibitor, we compared the amounts of protein yielded by four more *E. coli* purpose-designed strains. Thus, in addition to the BL21 (DE3) strain, the following commercially available strains were experimented with (all with various design specifics conditioned by their intended use in the context of production of recombinant proteins). Origami strain 2 (DE3), with mutations in the genes of thioredoxin reductase (trxB) and glutathione reductase (gor), which promote formation of disulfide bonds and correct folding of some recombinant proteins in the cytoplasm of bacterial cells. BL21 Star (DE3) strain, with a mutation in the gene encoding ribonuclease E (*rne131*), which improves stability of the target gene's mRNA and may boost production of recombinant proteins. It was also interesting to try the Rosetta (DE3) pLysS strain, which can produce proteins without codon optimization to *E. coli* and also allows reducing background expression from the T7 promoter by additional expression of the T7 lysozyme gene, a natural inhibitor of DNA-dependent RNA polymerase of the T7 phage, which is of particular importance in the context of production of proteins potentially toxic to bacteria. Finally, the *E. coli* KRX strain, with a chromosomal copy of the T7 DNA-dependent RNA polymerase gene controlled by the rhamnose promoter, which governs basal expression better, and is also well titrated by rhamnose while producing high amounts of recombinant proteins, as ensured by the T7 promoter [13].

To see how selection of the strain affects production of the soluble form of the inhibitor, we continued working with the variants that showed best results at the previous stage, i.e., pET28a-based vectors encoding chimeric proteins, with fusion polypeptides MBP and TIG. As mentioned above, all comparative experiments with these strains implied bacterial culture incubation at 16 °C. Ultimately, it was the KRX strain that delivered the best yield (Fig. 3). The possible reason behind this result is a more suitable mode of expression by the T7 DNA-dependent RNA polymerase gene controlled by the rhamnose promoter, which refines the dose of T7 DNA-dependent RNA polymerase accumulated in cells; accordingly, the recombinant ribonuclease inhibitor gene expression is

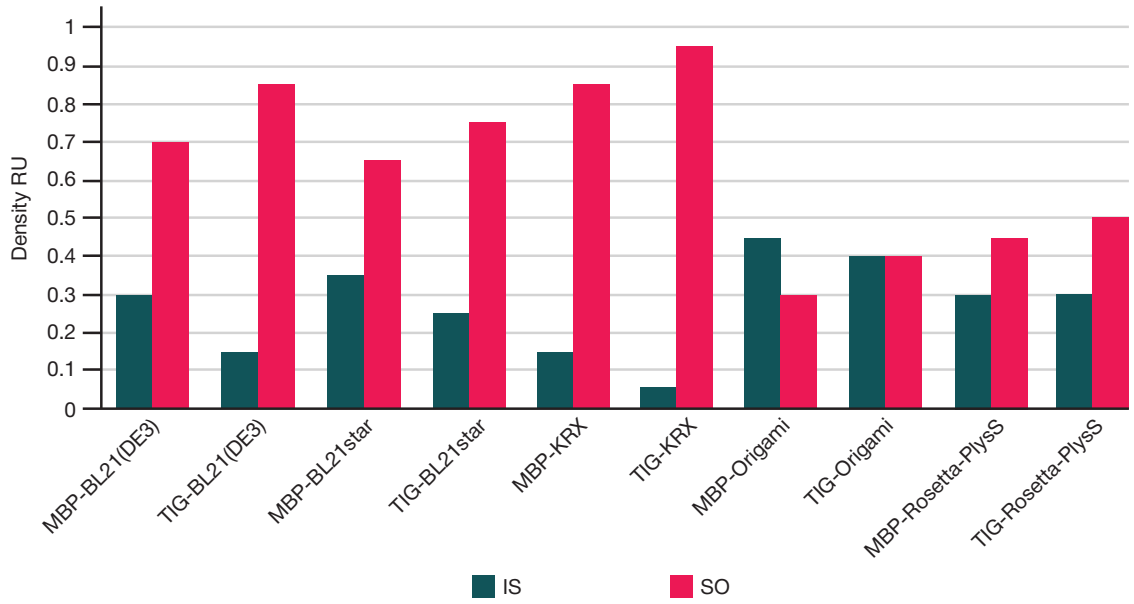

Fig. 3. Selection of the *E. coli* strain optimal for production of the recombinant ribonuclease inhibitor in soluble form, using vectors encoding a chimeric protein consisting of combinations of a ribonuclease inhibitor and two fusion polypeptides (MBP, TIG). IS is the relative level of insoluble fraction, shown in red. SO is the the level produced soluble ribonuclease inhibitor, shown in blue. Density RU are densitometry of PAAG results shown in relative units

not "overloaded," as in the case of direct induction of the T7 promoter with IPTG of lacUV5 promoter, when it is virtually impossible to weaken expression by titration. The BL21 (DE3) and BL21 Star (DE3) strains are also good options for the recombinant ribonuclease inhibitor production when combined with the selected fusion polypeptides and cultivated at low temperatures, but their yield of the soluble product is 10–15% (depending on the polypeptide) lower than that of the KRX strain. The remaining two strains performed considerably worse, with Origami 2 (DE3) being the outsider, probably due to its specific mutations designed to create intracellular conditions for the formation of disulfide bridges. Another possible reason for the poor results is the lower amount of biomass produced by these strains compared to KRX, BL21 (DE3) and BL21 Star (DE3), which may apparently be attributed to the introduced mutations affecting viability and metabolic processes in the cells. Thus, the second stage of the work revealed that in the context of production of the soluble form of ribonuclease inhibitor, the best "strain — fusion polypeptide" combinations are *E. coli* KRX-MBP and *E. coli* KRX-TIG.

There is one more tool used against formation of insoluble aggregates in production of recombinant proteins in *E. coli*: cellular chaperone genes co-expression, which helps proteins to adopt a native conformation. We tested the efficacy of this tool on chaperones *dnaK*, *dnaJ*, *drpE*, *groES* and *groEL*. Earlier, researchers reported a positive effect of some chaperones on the production of functional ribonuclease inhibitor [14, 15]. We decided to test them in the best combinations of the KRX *E. coli* strain and fusion polypeptide MBP, TIG, PpiB. This experiment revealed that the maximum effect — about 10% uptick of the target protein yield — was achieved against co-expression of genes of *dnaK*, *dnaJ*, *grpE* (Figure 4). An earlier study mentioned that GroELS chaperone positively influenced production of the soluble form of ribonuclease inhibitor [12]; in this regard, the likely reason behind the differences with our findings is the use of different strains.

Thus, the highest yielding ribonuclease inhibitor production combination identified in this work includes the KRX *E. coli* strain, TIG and MBP fusion polypeptides, co-expressing *dnaK*, *dnaJ*, *grpE* chaperone genes, and cultivation temperature of 16 °C. To accurately assess the yield of the target protein given by the tested combinations, we used affinity chromatography on a metal chelate carrier and subsequent proteolysis reaction with TEV protease. The total amount of soluble ribonuclease inhibitor was 60 mg/l in case of *E. coli* KRX-TIG-*dnaK*, *dnaJ*, *grpE*, and 45 mg/l for *E. coli* KRX-MBP-*dnaK*, *dnaJ, grpE*.

DISCUSSION

The rapid development of therapeutic applications of synthetic mRNAs underpins the need for preparative amounts of proteins required to produce large amounts of RNA *in vitro* [16, 17]. Apart from the enzymes that synthesize and modify RNA *in vitro*, another important component of the reaction mixture is ribonuclease inhibitor, a protein that prevents degradation of the RNA molecules caused by ribonucleases, which is crucial for medicines. Ribonuclease inhibitor is also widely used in research tasks related to the synthesis of RNA, cDNA, and *in vitro* translation. The production of this protein in recombinant *E. coli* strains is complicated by the specificity of its structure with abundant cysteine residues, and the tendency to aggregate in bacterial cells. Previous attempts at production of the soluble form of ribonuclease inhibitor in *E. coli* require special conditions that significantly complicate the technological process and make it costly to scale [11, 12]. This work aimed to find the ribonuclease inhibitor production conditions and method that would deliver large yields and be cost-effective from the technological point of view.

As the first stage, we screened a number of fusion peptides that could increase the proportion of soluble ribonuclease inhibitor produced in *E. coli*. Previous studies have shown that polypeptides can significantly increase the yield of soluble form of recombinant proteins, including ribonuclease inhibitors [4, 18]. The ability to improve solubility of various recombinant proteins stems from the formation of chimeric molecules, the fusion part of which (polypeptide) can later be removed by proteolysis [9, 19]. We tested nine different fusion polypeptides: MBP, TIG, TSF, FH8, PpiB, YrhB, SUMO, TRX, GST; they differ significantly in their physico-chemical properties. Additionally, we tested them in two expression systems, one based on the commercially available plasmid pET28a (target gene expression controlled by a strong T7 promoter), and another on the pSOL

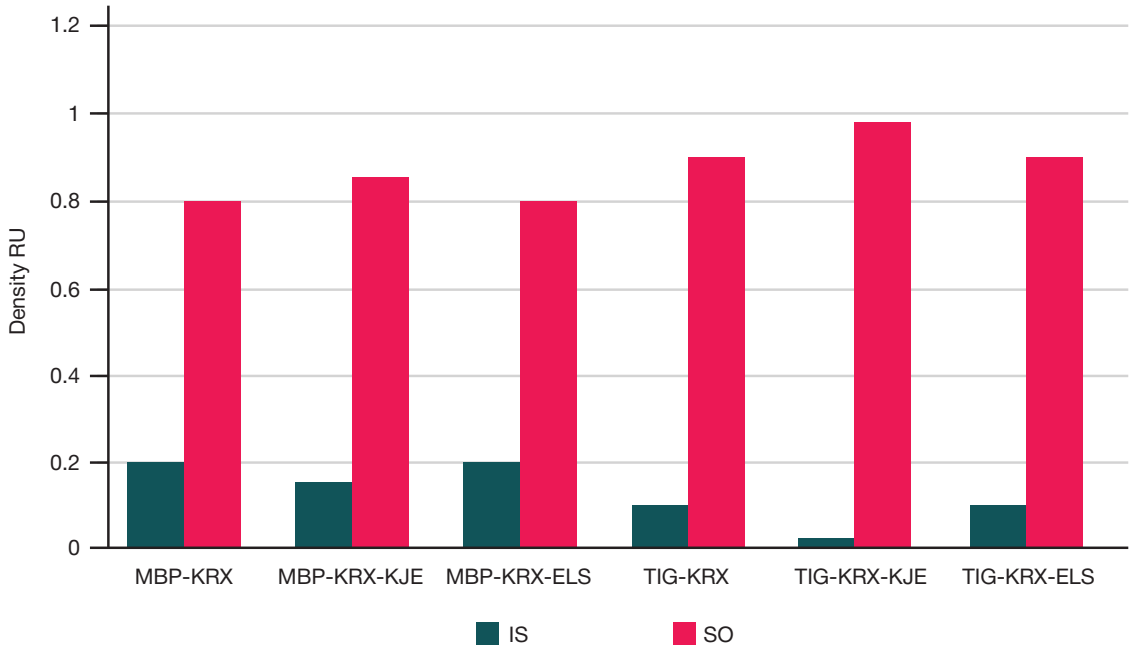

Fig. 4. Effect of additional gene co-expression, cellular chaperones *dnaK*, *dnaJ*, *grpE* (shown as KJE), *groES* and *groEL* (shown as ELS), on production of the recombinant ribonuclease inhibitor in the KRX *E. coli* strain at 16 °C. IS is the relative level of insoluble fraction, shown in red. SO is the the level produced soluble ribonuclease inhibitor, shown in blue. Density RU are densitometry of PAAG results shown in relative units

vector (expression controlled by a rhamnose promoter). The best results were ensured by MBP, TIG, and PpiB, with the yield highest in the systems utilizing MBP and TIG, and that with PpiB performing considerably worse (Fig. 2). Overall, all the experiments of this work have shown that larger polypeptides, such as MBP (42 kDa) and TIG (49 kDa), provide more of the final target product than fusions with smaller molecular weight, like PpiB (19 kDa). Comparing the yield of target products at the level of vector lines (pET28a and pSol, in this study), the unambiguous system of choice for production of ribonuclease inhibitor is that where a strong T7 promoter controls target gene expression (Fig. 2).

Having established that the most productive vector is that based on pET28a, encoding chimeric ribonuclease inhibitor variants with MBP and TIG fusion polypeptides, we moved to the next stage of optimization: selection of the suitable strain of *E. coli* from several commercially available options. For this purpose, we compared the ribonuclease inhibitor production capacity of BL21 (DE3), Origami 2 (DE3), BL21 Star (DE3), Rosetta (DE3) pLysS, and KRX *E. coli* strains, all of which are designed for production of recombinant proteins, but have certain specifics, which enables the comparison and allows selecting that which maximizes production of the soluble form ribonuclease inhibitor [20]. Under the conditions of this study, the highest ribonuclease inhibitor yield was delivered by the KRX *E. coli* strain (Fig. 3), likely because of the rhamnose promoter control over the T7 gene of DNA-dependent RNA polymerase, since this promoter has a low basal activity level and is better titrated by L-rhamnose. Apparently, this design feature allows finding the optimal balance of conditions for bacterial production of toxic or aggregation-prone recombinant proteins, while also ensuring a high yield of the target protein by reducing the amount of protein loss in inclusion bodies, which is common for production in strains with the chromosomal copy of T7 DNA-dependent RNA polymerase controlled by the lacUV5 promoter and induced by IPTG [21].

Finally, having established that the best "strain — fusion polypeptide" combinations are *E. coli* KRX-MBP and *E. coli* KRX-TIG, we decided to investigate the effect of the additional cellular chaperone genes co-expression on the yield of the target protein. For this purpose, we used a commercial kit (Takara; USA) that includes vectors carrying the genes of chaperones *dnaK*, *dnaJ*, *grpE*, *groES* and *groEL*, which, when produced additionally in the *E. coli* cells, often help "problematic" recombinant proteins to adopt the correct conformation, thus reducing the formation of insoluble aggregates [14, 15]. For recombinant ribonuclease inhibitor, the positive effect of groES and groEL on the protein production under anaerobic conditions has already been shown [12], but this approach is not always convenient when scaling technological processes to produce larger amounts of recombinant proteins. Interestingly, in our experimental conditions, aerobic cultivation of *E. coli* at a reduced temperature (16 °C), the best ribonuclease inhibitor yields were given by two combinations: a) MBP fusion polypeptide, *dnaK*, *dnaJ*, *grpE*, chaperone genes co-expression, KRX *E. coli* strain, and b) TIG fusion polypeptide, *dnaK*, *dnaJ*, *grpE* chperone genes co-expression, KRX *E. coli* strain (Fig. 4). To accurately quantify recombinant ribonuclease inhibitor in such combinations, we performed affinity purification and then compared the the yield of the target product. The first combination produced 45 mg/l. However, the maximum yield of the final product, that is, the soluble form of ribonuclease inhibitor, was achieved in aerobic cultivation of the KRX *E. coli* strain at a reduced temperature (16 °C), transformed by the expression vector based on pET28a with a codon optimized ribonuclease inhibitor gene, in combination with the TIG fusion polypeptide gene, against the background of *dnaK*, *dnaJ*, *grpE* chaperone genes co-expression. This combination gives a yield of 60 mg/l when culturing bacteria in flasks on a standard LB medium, which is significantly higher than the results achieved earlier, and also easier from the bacteria cultivation perspective [4, 11, 12, 22].

The results of our study confirm the need for an integrated approach to the search for optimal conditions of production of aggregation-prone recombinant proteins in *E. coli*, especially when the goal is to scale up the process of synthesis of enzymes, therapeutic proteins, cytokine preparations or antigens for vaccines [23].

CONCLUSIONS

This work shows that a systemic approach to identification of conditions and components for production of enzymes and therapeutic recombinant proteins in *E. coli*, given that they are prone to aggregation and form inclusion bodies under normal bacterial cultivation conditions, allows finding combinations delivering significantly higher yields of the target protein in soluble form. Moreover, it is possible to find the balance of conditions and components needed to establish production at semi-industrial and industrial scales, and to minimize protein loss to aggregation. It should be noted that the final ribonuclease inhibitor production protocol compiled in this study can be optimized further by incorporating the experience of earlier works and opting for a nutrient media other than the standard LB, as well as introduction of cultivation in a fermenter, which will further increase the final product yield. However, even in its present form, the protocol can support semi-industrial production of this protein in preparative quantities for further use in enzymatic RNA synthesis *in vitro*.

References

1. Moenner M, Vosoghi M, Ryazantsev S, Glitz DG. Ribonuclease inhibitor protein of human erythrocytes: characterization, loss of activity in response to oxidative stress, and association with Heinz bodies. Blood Cells, Molecules, and Diseases. 1998; 24 (2): 149–64. DOI: 10.1006/bcmd.1998.0182.
2. Klink TA, Vicentini AM, Hofsteenge J, Raines RT High-level soluble production and characterization of porcine ribonuclease inhibitor. Protein expression and purification. 2001; 22 (2): 174–79. DOI: 10.1006/prep.2001.1422.
3. Kobe B, Deisenhofer J. Mechanism of ribonuclease inhibition by ribonuclease inhibitor protein based on the crystal structure of its complex with ribonuclease A. Journal of molecular biology. 1996; 264 (5): 1028–43. DOI: 10.1006/jmbi.1996.0694.
4. Guo W, Cao L, Jia Z, Wu G, Li T, Lu F, et al. High level soluble production of functional ribonuclease inhibitor in Escherichia coli by fusing it to soluble partners. Protein expression and purification. 2011; 77 (2): 185–92. DOI: 10.1016/j.pep.2011.01.015.
5. Dickson KA, Haigis MC, Raines RT. Ribonuclease inhibitor: structure and function. Progress in nucleic acid research and molecular biology. 2005; 80: 349–74. DOI: 10.1016/S0079-6603(05)80009-1.
6. Fominaya JM, Hofsteenge J. Inactivation of ribonuclease inhibitor by thiol-disulfide exchange. Journal of Biological Chemistry. 1992; 267 (34): 24655–60.
7. Kobe B, Kajava AV. The leucine-rich repeat as a protein recognition motif. Current opinion in structural biology. 2001; 11 (6): 725–32. DOI: 10.1016/s0959-440x(01)00266-4.
8. Blackburn P. Ribonuclease inhibitor from human placenta: rapid purification and assay. Journal of Biological Chemistry. 1979; 254 (24): 12484–7.
9. Rosano GL, Ceccarelli EA. Recombinant protein expression in Escherichia coli: advances and challenges. Frontiers in microbiology. 2014; 5: 172. DOI: 10.3389/fmicb.2014.00172.
10. Lee FS, Vallee BL. Expression of human placental ribonuclease inhibitor in Escherichia coli. Biochemical and biophysical research communications. 1989; 160 (1): 115–20. DOI: 10.1016/0006-291x(89)91628-8.
11. Šiurkus J, Neubauer P. Reducing conditions are the key for efficient production of active ribonuclease inhibitor in Escherichia coli. Microbial Cell Factories. 2011; 10 (1): 1–15. DOI: 10.1186/1475-2859-10-31.
12. Šiurkus J, Neubauer P. Heterologous production of active ribonuclease inhibitor in Escherichia coli by redox state control and chaperonin coexpression. Microbial cell factories. 2011; 10: 1–11. DOI: 10.1186/1475-2859-10-65.
13. Ohana RF, Encell LP, Zhao K, Simpson D, Slater MR, Urh M, et al. HaloTag7: A genetically engineered tag that enhances bacterial expression of soluble proteins and improves protein purification. Protein Expression and Purification. 2009; 68 (1): 110–20. DOI: 10.1016/j.pep.2009.05.010.
14. Nishihara K, Kanemori M, Kitagawa M, Yanagi H, Yura, T. Chaperone coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in Escherichia coli. Appl. Environ Microbiol. 1998; 64: 1694–99. DOI: 10.1128/AEM.64.5.1694-1699.
15. Nishihara K, Kanemori M, Yanagi H, Yura T. Overexpression of trigger factor prevents aggregation of recombinant proteins in Escherichia coli. Appl. Environ Microbiol. 2000; 66: 884–89. DOI: 10.1128/AEM.66.3.884-889.2000.
16. Zaharova MV, Nagornyh MO. Poluchenie bakterial'nogo producenta DNK-zavisimoj RNK-polimerazy bakteriofaga T7 dlja sinteza RNK in vitro. Mezhdunarodnyj zhurnal prikladnyh i fundamental'nyh issledovanij. 2022; 12: 9–14. DOI: 10.17513/mjpfi.13476. Russian.
17. Fuchs AL, Neu A, Sprangers R. A general method for rapid and cost-efficient large-scale production of 5' capped RNA. RNA. 2016; 22 (9): 1454–66. DOI: 10.1261/rna.056614.116.
18. Ortega C, Oppezzo P, Correa A. Overcoming the Solubility Problem in E. coli: Available Approaches for Recombinant Protein Production. Methods Mol Biol. 2022; 2406: 35–64. DOI: 10.1007/978-1-0716-1859-2_2.
19. Jia B, Jeon CO. High-throughput recombinant protein expression in Escherichia coli: current status and future perspectives. Open biology. 2016; 6 (8): 160196. DOI: 10.1098/rsob.160196.
20. Morão LG, Manzine LR, Clementino LOD, Wrenger C, Nascimento AS. A scalable screening of E. coli strains for recombinant protein expression. PLOS ONE 2022; 17 (7): e0271403. DOI: 10.1371/journal.pone.0271403.
21. Zhang ZX, Nong FT, Wang YZ, Yan CX, Gu Y, Song P, et al. Strategies for efficient production of recombinant proteins in Escherichia coli: alleviating the host burden and enhancing protein activity. Microb Cell Fact. 2022; 21 (191). DOI: 10.1186/s12934-022-01917-y.
22. Flachner B, Dobi K, Benedek A, Cseh S, Lőrincz Z, Hajdú I. Robust Recombinant Expression of Human Placental Ribonuclease Inhibitor in Insect Cells. Biomolecules. 2022; 12 (2): 273. DOI: 10.3390/biom12020273.
23. Mital S, Christie G, Dikicioglu D. Recombinant expression of insoluble enzymes in Escherichia coli: a systematic review of experimental design and its manufacturing implications. Microb Cell Fact. 2021; 20 (1): 208. DOI: 10.1186/s12934-021-01698-w.

Литература

1. Moenner M, Vosoghi M, Ryazantsev S, Glitz DG. Ribonuclease inhibitor protein of human erythrocytes: characterization, loss of activity in response to oxidative stress, and association with Heinz bodies. Blood Cells, Molecules, and Diseases. 1998; 24 (2): 149–64. DOI: 10.1006/bcmd.1998.0182.
2. Klink TA, Vicentini AM, Hofsteenge J, Raines RT High-level soluble production and characterization of porcine ribonuclease inhibitor. Protein expression and purification. 2001; 22 (2): 174–79. DOI: 10.1006/prep.2001.1422.
3. Kobe B, Deisenhofer J. Mechanism of ribonuclease inhibition by

ribonuclease inhibitor protein based on the crystal structure of its complex with ribonuclease A. Journal of molecular biology. 1996; 264 (5): 1028–43. DOI: 10.1006/jmbi.1996.0694.
4. Guo W, Cao L, Jia Z, Wu G, Li T, Lu F, et al. High level soluble production of functional ribonuclease inhibitor in Escherichia coli by fusing it to soluble partners. Protein expression and purification. 2011; 77 (2): 185–92. DOI: 10.1016/j.pep.2011.01.015.
5. Dickson KA, Haigis MC, Raines RT. Ribonuclease inhibitor: structure and function. Progress in nucleic acid research and molecular biology. 2005; 80: 349–74. DOI: 10.1016/S0079-6603(05)80009-1.
6. Fominaya JM, Hofsteenge J. Inactivation of ribonuclease inhibitor by thiol-disulfide exchange. Journal of Biological Chemistry. 1992; 267 (34): 24655–60.
7. Kobe B, Kajava AV. The leucine-rich repeat as a protein recognition motif. Current opinion in structural biology. 2001; 11 (6): 725–32. DOI: 10.1016/s0959-440x(01)00266-4.
8. Blackburn P. Ribonuclease inhibitor from human placenta: rapid purification and assay. Journal of Biological Chemistry. 1979; 254 (24): 12484–7.
9. Rosano GL, Ceccarelli EA. Recombinant protein expression in Escherichia coli: advances and challenges. Frontiers in microbiology. 2014; 5: 172. DOI: 10.3389/fmicb.2014.00172.
10. Lee FS, Vallee BL. Expression of human placental ribonuclease inhibitor in Escherichia coli. Biochemical and biophysical research communications. 1989; 160 (1): 115–20. DOI: 10.1016/0006-291x(89)91628-8.
11. Šiurkus J, Neubauer P. Reducing conditions are the key for efficient production of active ribonuclease inhibitor in Escherichia coli. Microbial Cell Factories. 2011; 10 (1): 1–15. DOI: 10.1186/1475-2859-10-31.
12. Šiurkus J, Neubauer P. Heterologous production of active ribonuclease inhibitor in Escherichia coli by redox state control and chaperonin coexpression. Microbial cell factories. 2011; 10: 1–11. DOI: 10.1186/1475-2859-10-65.
13. Ohana RF, Encell LP, Zhao K, Simpson D, Slater MR, Urh M, et al. HaloTag7: A genetically engineered tag that enhances bacterial expression of soluble proteins and improves protein purification. Protein Expression and Purification. 2009; 68 (1): 110–20. DOI: 10.1016/j.pep.2009.05.010.
14. Nishihara K, Kanemori M, Kitagawa M, Yanagi H, Yura, T. Chaperone coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in Escherichia coli. Appl. Environ Microbiol. 1998; 64: 1694–99. DOI: 10.1128/AEM.64.5.1694-1699.
15. Nishihara K, Kanemori M, Yanagi H, Yura T. Overexpression of trigger factor prevents aggregation of recombinant proteins in Escherichia coli. Appl. Environ Microbiol. 2000; 66: 884–89. DOI: 10.1128/AEM.66.3.884-889.2000.
16. Захарова М. В., Нагорных М. О. Получение бактериального продуцента ДНК-зависимой РНК-полимеразы бактериофага Т7 для синтеза РНК in vitro. Международный журнал прикладных и фундаментальных исследований. 2022; 12: 9–14. DOI: 10.17513/mjpfi.13476.
17. Fuchs AL, Neu A, Sprangers R. A general method for rapid and cost-efficient large-scale production of 5' capped RNA. RNA. 2016; 22 (9): 1454–66. DOI: 10.1261/rna.056614.116.
18. Ortega C, Oppezzo P, Correa A. Overcoming the Solubility Problem in E. coli: Available Approaches for Recombinant Protein Production. Methods Mol Biol. 2022; 2406: 35–64. DOI: 10.1007/978-1-0716-1859-2_2.
19. Jia B, Jeon CO. High-throughput recombinant protein expression in Escherichia coli: current status and future perspectives. Open biology. 2016; 6 (8): 160196. DOI: 10.1098/rsob.160196.
20. Morão LG, Manzine LR, Clementino LOD, Wrenger C, Nascimento AS. A scalable screening of E. coli strains for recombinant protein expression. PLOS ONE 2022; 17 (7): e0271403. DOI: 10.1371/journal.pone.0271403.
21. Zhang ZX, Nong FT, Wang YZ, Yan CX, Gu Y, Song P, et al. Strategies for efficient production of recombinant proteins in Escherichia coli: alleviating the host burden and enhancing protein activity. Microb Cell Fact. 2022; 21 (191). DOI: 10.1186/s12934-022-01917-y.
22. Flachner B, Dobi K, Benedek A, Cseh S, Lőrincz Z, Hajdú I. Robust Recombinant Expression of Human Placental Ribonuclease Inhibitor in Insect Cells. Biomolecules. 2022; 12 (2): 273. DOI: 10.3390/biom12020273.
23. Mital S, Christie G, Dikicioglu D. Recombinant expression of insoluble enzymes in Escherichia coli: a systematic review of experimental design and its manufacturing implications. Microb Cell Fact. 2021; 20 (1): 208. DOI: 10.1186/s12934-021-01698-w.