JABET
Journal of Advanced Biotechnology and Experimental Therapeutics

ORIGINAL ARTICLE

J Adv Biotechnol Exp Ther. 2023 Sep; 6(3): 610-621
eISSN: 2616-4760, https://doi.org/10.5455/jabet.2023.d153
Published by www.bsmiab.org

Cloning and expression of recombinant purine nucleoside phosphorylase in the methylotrophic yeast *Pichia pastoris*

Jaloliddin Abdurakhmanov[1,*], Sobirdjan Sasmakov[1], Shukhrat Khasanov[1], Oybek Ashirov[1], Farkhod Eshboev[1], Khayotjon Dolimov[1], Saodat Gaynazarova[1], Tulkin Sadullaev[1], Oygul Yarilkaganova[1], Khusan Nasriddinov[1], Asadali Baymirzaev[1], Artyom Makhnyov[1], Shakhnoz Azimov[1]

[1]S.Yu. Yunusov Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan, Mirzo Ulugbek Str. 77, 100170, Tashkent, Uzbekistan

***Corresponding author**
Jaloliddin Abdurakhmanov, PhD
S.Yu. Yunusov Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan, Mirzo Ulugbek Str. 77, 100170, Tashkent, Uzbekistan
Email: jaloliddin0919@mail.ru

Academic editor
Mohammad Nazrul Islam, PhD
Sher-e-Bangla Agricultural University, Bangladesh

Article info
Received: 14 April 2023
Accepted: 12 June 2023
Published: 18 July 2023

Keywords
Enzymatic hydrolysis, Inosine, Modified nucleosides, *Pichia pastoris*, Purine nucleoside phosphorylase

ABSTRACT

Purine nucleoside phosphorylase (PNP) is an enzyme involved in biosynthetic pathway of purine nucleosides. Purine nucleoside phosphorylase catalyzes the cleavage of the glycosidic bond of ribo- or deoxyribonucleosides to form the purine base and deoxyribose- or ribose-1-phosphate. The reversible reaction catalyzed by recombinant PNP of *E. coli* (*Ec*PNP) has been successfully used for the synthesis of nucleoside analogues. The aim of this study was to clone and expression of recombinant PNP in *Pichia pastoris*. For expression of recombinant *Ec*PNP in *Pichia pastoris*, a new recombinant plasmid DNA pPICZαA-PNP (4256 bp) containing the deoD gene (720 bp) amplified from *E. coli* strain RKMUz-221 has been cloned. The substrate specificity of the recombinant *Ec*PNP expressed in yeast cells was studied by hydrolysis of inosine (9-beta-D-ribofuranosylhypoxanthine) to hypoxanthine and 2-deoxy-β-D-ribofuranosyl. The fraction containing the obtained recombinant EcPNP (≈28 kDa, as determined by SDS-PAGE) demonstrates significant hydrolytic activity, resulting in up to a 51% hydrolysis of inosine within a 5 h timeframe. These findings suggest that the recombinant enzyme holds a promise for applications in enzymatic transglycosylation of purine-type nucleosides.

INTRODUCTION

Nucleoside phosphorylases (NPs) are widely applied for the synthesis of modified nucleosides, which have used as potential anticancer, antiviral, and antibacterial drugs [1-4]. Based on their substrate spectrum, NPs are classified into purine nucleoside phosphorylases (PNP, EC 2.4.2.1) and pyrimidine nucleoside phosphorylases (PyNP, EC 2.4.2.2). Both catalyze the reversible phosphorolysis (transglycosylation) of nucleosides to nucleobases and α-D-pentofuranose-1-phosphates [5-6]. Enzymatic modification of nucleosides promises increased regioselectivity and exquisite control of the transglycosylation and is a better alternative to the commonly used chemical synthesis [6-7].

In recent years, *E. coli* purine nucleoside phosphorylases have attracted increasing interest as a biocatalyst for the synthesis of purine-type nucleosides [8-10]. PNP catalyzes the reversable hydrolysis of the glycosidic bond of pentofuranoses in the presence of inorganic phosphate [10-11].

In transglycosylation reactions, bacterial nucleoside phosphorylases are used either as part of cell lysates (raw) or as recombinant enzymes obtained by genetic engineering. In this regard, obtaining new strains of microorganisms producing recombinant enzymes with a high yield is relevant. *Pichia pastoris* is one of the advanced expression systems

used in biotechnology, characterized by a high yield of recombinant proteins compared to other expression systems and the absence of endotoxins/pyrogens. Therefore, the aim of this study was to clone and express recombinant purine nucleoside phosphorylase in *Pichia pastoris* as a producer on an industrial scale [12-14].

MATERIALS AND METHODS

Chemicals

Restriction enzymes (FastDigest *EcoRI*, FastDigest *XbaI* and FastDigest *SacI*) and T4 DNA ligase used in DNA clonning were purchased from Intivrogen (Thermo Scientific™, USA). Inosine (Ino), hypoxanthine (6-hydroxypurine), D-ribose, and purine nucleoside phosphorylase (10 U/mg) were purchased from Sigma (Germany). All other chemicals were purchased from Sigma in the highest purity.

Escherichia coli genomic DNA extraction

Bacterial genomic DNA was isolated from pure cultures of *Escherichia coli* strain RKMUz – 221 (the strain was obtained from the collection of industrial microorganisms of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan) by using the previously published protocol [15]. Bacterial cells were grown in liquid nutrient medium (LB Broth Miller, Invitrogen) at 37°C for 12 h. Isolation was carried out in a 1.5 ml tube containing 1 ml of the overnight *E. coli* culture. Centrifuged at max speed for 1min to pellet the cells. Remove as much of the supernatant as we can without disturbing the cell pellet. The cell pellet resuspended in 600 µl lysis buffer (9.34 ml TE buffer pH 8, 600 µl of 10% SDS, 60 µl of proteinase K (20 mg/ml)) and then vortexed. The mixture was incubated 1 h at 37°C. After that, lysate treated an equal volume 2 times using phenol/chloroform without vertex and 1 time chloroform. After centrifuged at max speed for 5 min the upper aqueous phase was carefully transferred to a new tube. For precipitating the DNA, it was added 3 volumes of cold 96% ethanol and mixed gently. Finally, 1µl of samples were used directly for PCR in 20 µl reactions.

Preparation of transfer vector and cDNA (gene) for ligation

1 µg of the transfer vector pPICZαA was sequentially treated using FastDigest *EcoRI* and FastDigest *XbaI* restrictases. Linear DNA 3536 bp was isolated after 0.7% agarose gel electrophoresis using PureLink™ Quick Gel Extraction Kit [16].

Purine nucleoside phosphorylase cDNA (deoD gene, insert), size of 720 bp was amplified by PCR using as a template genomic DNA isolated from cells of *Escherichia coli* strain RKMUz–221 using the following primers:

Forward – 5′-ccaagaattcatggctaccccacacattaatgc-3′,

Reverse – 5′-cttgtctagatattactctttatcgcccagcagaac-3′

After electrophoresis in 1% agarose gel the amplificon (insert) was purified using PureLink™ Quick Gel Extraction Kit [16] and treated with the same enzymes which used for the transfer vector.

Ligation of the vector with cDNA (purine nucleoside phosphorylase gene)

Ligation of the pPICZαA vector with the purine nucleoside phosphorylase gene (*E. coli* PNP) was carried out in a volume of 10 µl in a molar ratio of 1:3, accordingly, using recombinant the phage T4 DNA ligase (Thermo Scientific™) [17-18].

Transformation of *E. coli* TOP10 with a ligation mixture

After precipitation with the ligation mixture, the pellet (recombinant DNA) was dissolved in 10 µl of de-ionized water. Dissolved DNA was transformed into electrocompetent plasmid-free strain *E. coli* TOP10 cells (Invitrogen) according to the method [19].

Identification of recombinant clones containing pPICZαA- PNP

The molecular weight of plasmid DNA isolated from clones obtained after transformation with a ligation mixture was determined using 0.8% agarose gel electrophoresis. Clones containing DNA with a length of 4256 bp (deoD gene + pPICZαA transfer vector fragment) were investigated by PCR analysis. PCR amplicons were analyzed by electrophoresis in 1% agarose gel [20].

Sequencing of cloned cDNA

Recombinant cDNAs were sequenced as described [21] in DNA Sequencing with the SeqStudio EDDU protocols using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). Three identical cycle sequencing reactions (containing 50 ng of the template DNA); 5 pmol of the gene-specific primers; 4 µL of BigDye Terminator v3.1 Ready reaction Mix; and deionized water to a final vol of 10 µL were subjected to an initial denaturation step of 96°C for 2 min, followed by 30 cycles of 96°C for 20 s, 60°C for 10 s, 65°C for 4 min in a QuantStudio PCR System (Applied Biosystems, Thermo Fisher Scientific, USA). Sequencing purification was performed using ZR DNA Sequencing Clean-Up Kit as described previously [22]. Automated sequencing traces were obtained in SeqStudio Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA).

Accession numbers for target gene nucleotide sequences

The nucleotide sequences of the PNP (deoD gene) presented in this study have been entered to the National Centre for Biotechnology Information (NCBI) as GenBank accession number OQ133467.1.

Transformation of the pPICZαA-PNP vector into *Pichia pastoris* GS115

The recombinant pPICZαA-PNP vector was linearized with FastDigest *SacI* and the mixture was precipitated with absolute ethanol in the presence of 3M Sodium acetate pH 5.2 to purify and concentrate the DNA. The purified linear DNA vector was transformed into *Pichia pastoris* GS115 yeast using an Electroporator 2510 (Eppendorf, USA). Then, the yeast transformants was grown in YPD selection medium containing the antibiotic Zeocin™ (100 µg/mL).

Expression, purification, and SDS-PAGE identification of recombinant *Ec*PNP

To induce *Ec*PNP expression, *Pichia pastoris* GS115 transformants were inoculated in BMGY medium at 30°C with vigorous shaking (220 rpm), and then transferred to BMMY and incubated at 30°C with vigorous shaking (220 rpm) up to 96 h. Methanol (0.5-1%, v/v) was supplied to the culture medium once a day [23].

Extraction of the enzyme was carried out by destroying the yeast cells using a benchtop two-stage high pressure homogenizer (APV - 2000, SPX Flow, USA). The device can treat liquid samples at pressures up to 2000 bar. The two-stage (two times 1200 bar) was used after spin pellets for 1 min at 14.000 x g at 4°C and resuspend pellets in breaking buffer (Tris-Cl (1 M, pH 8.0) – 10mM, EDTA (0.5 M, pH 8.0) – 1mM, Triton X-100 - 2% (v/v), SDS (10%) - 1% (w/v), NaCl (5 M) - 100 mM, PMSF - 2mM) for the treatment of yeast suspensions. The suspension temperature at the exit of the homogenizer did not exceed 40°C. Treated samples were cooled to 15°C immediately at the outlet of the device using a water-ice bath. After that, the yeast culture liquid medium was added to the homogenate.

Homogenate was precipitated using gradient precipitation with 25%-65% ammonium sulphate to obtain primary purified purine nucleoside phosphorylase. The prepared solution in 65% ammonium sulphate was incubated for 4 h at 40C. The fraction containing the enzyme was separated by centrifugation at 14.000 x g at 4°C for 30 min. The collected pellets (enzyme fraction) were dissolved in BS (150 mM NaCl, 5.2 mM Na_2HPO_4, 1.7mM KH_2PO_4), pH 7.4.

The molecular weight was determined with 10% PAGE electrophoresis in the presence of Na dodecyl sulphate (SDS) according to the Laemmle method [24]. The protein concentration was determined by the Lowry method, using bovine serum albumin (BSA) as the standard [25].

Determination of *Ec*PNP expression

The expression of *Ec*PNP in *Pichia pastoris* yeast cells were determined by Purine Nucleoside Phosphorylase Activity Fluorometric Assay Kit (ab204706, Abcam PLC, UK) [26].

Enzymatic hydrolysis of inosine with obtained *Ec*PNP

Reaction mixture contained 3.5 mM KH_2PO_4, pH 8.0, 10 mM 9-beta-D-ribofuranosylhypoxanthine (99%, Sigma-Aldrich, Cat. I4125-100G) and 5 µl *Ec*PNP fraction (total protein cons. 15.8 mg/ml). The reaction mixtures were incubated at 50°C up to 5 h. Substrate and product quantities were determined using HPLC.

HPLC detection

The quantitative determination of the products was performed by HPLC analysis using a Shim-pack GIST C18 5 µm, 4.6 × 150 mm column (Shimadzu) under the following conditions: flow gradient (100-90% 10 mM KH_2PO_4 in H_2O and 0-10% MeCN) for 30 min, flow rate 0.5 mL/min, detection PDA at 254 nm. The retention times for the substrates and products are: 9-beta-D-ribofuranosylhypoxanthine (Inosine) ~ 20.41 min; 6-hydroxypurine (hypoxanthine) ~ 16.31 min.

RESULTS

Designing, cloning and transformation of the recombinant pPICZαA-PNP

Plasmid DNA pPICZαA-PNP (4256 bp) was created on the basis of the transfer vector pPICZαA (3593 bp), which was designed for intracellular expression of proteins in the yeast *Pichia pastoris*. Figure 1 shows the genetic maps of the cloned plasmid DNA pPICZαA-PNP.

The obtained clones were screened by PCR. Figure 2 shows the result of PCR analysis of recombinant clones. According to the results of PCR, the selected clones contain DNA fragments (gene) with a molecular weight of 720 bp (Figure 2). Thus, PCR indicated the presence of an insert (the PNP-deoD gene) in the recombinant plasmid.

Figure 3 shows a comparative restriction analysis of the recombinant plasmid pPICZαA-PNP and the original pPICZαA vector at a single restriction site of FastDigest *SacI*. After electrophoresis in 0.8% agarose gel, linear DNA was detected, the mass of which corresponds to theoretical calculations according to the physical maps of plasmids. At the same time, the difference between the sizes of the two plasmids was evident.

Linearized vector plasmid with FastDigest *SacI* was transformed into *Pichia pastoris* GS115 yeast using a Electroporator 2510 and the yeast transformants was grown in YPD selection medium containing the antibiotic Zeocin™ (100 μg/mL).

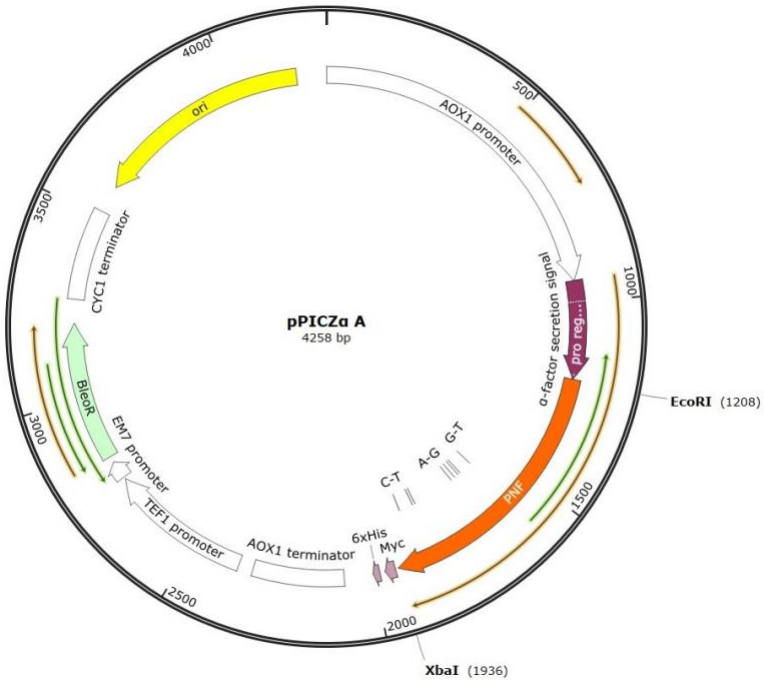


Figure 1. Physical map of the recombinant plasmid pPICZαA-PNP.

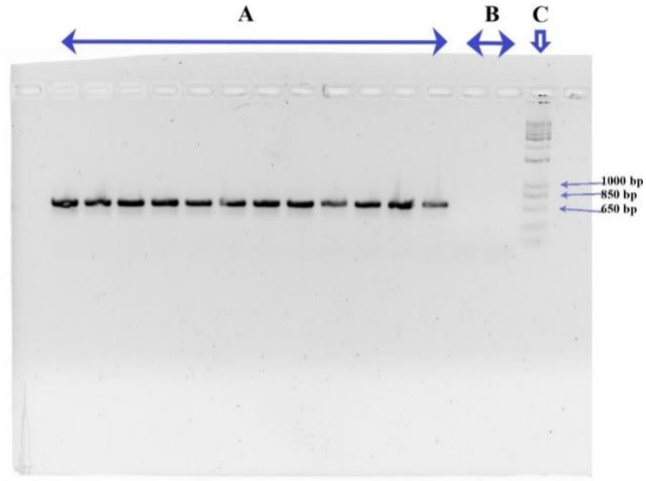


Figure 2. PCR analysis of the recombinant *E. coli* Top10 clones: (A) Clones; (B) K- (pPICZαA); (C) DNA marker.

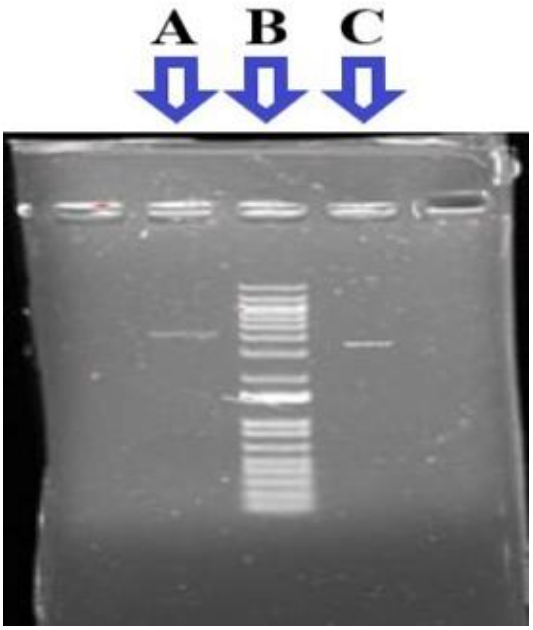


Figure 3. Restriction analysis of the recombinant plasmid pPICZαA-PNP and original plasmid by identical restriction site: (A) pPICZαA-TP/SacI (4256 bp); (B) pPICZαA/SacI (3593 bp); (C) DNA Marker.

cDNA (gene) sequencing

Nucleotide sequencing by Sanger was used to verify cloned *E. coli* strain RKMUz - 221 PNPase. The results showed that the purine nucleoside phosphorylase gene has 98.47% sequence similarity with *Escherichia coli* strain BM28 (GenBank accession no. CP10280.1). There were eight different mutations discovered in PNP of *E. coli* strain RKMUz – 221 and all of which were single substitutions (T>G, G>A, G>A, C>T, T>G, T>C, C>A and C>T) (Figure 4).

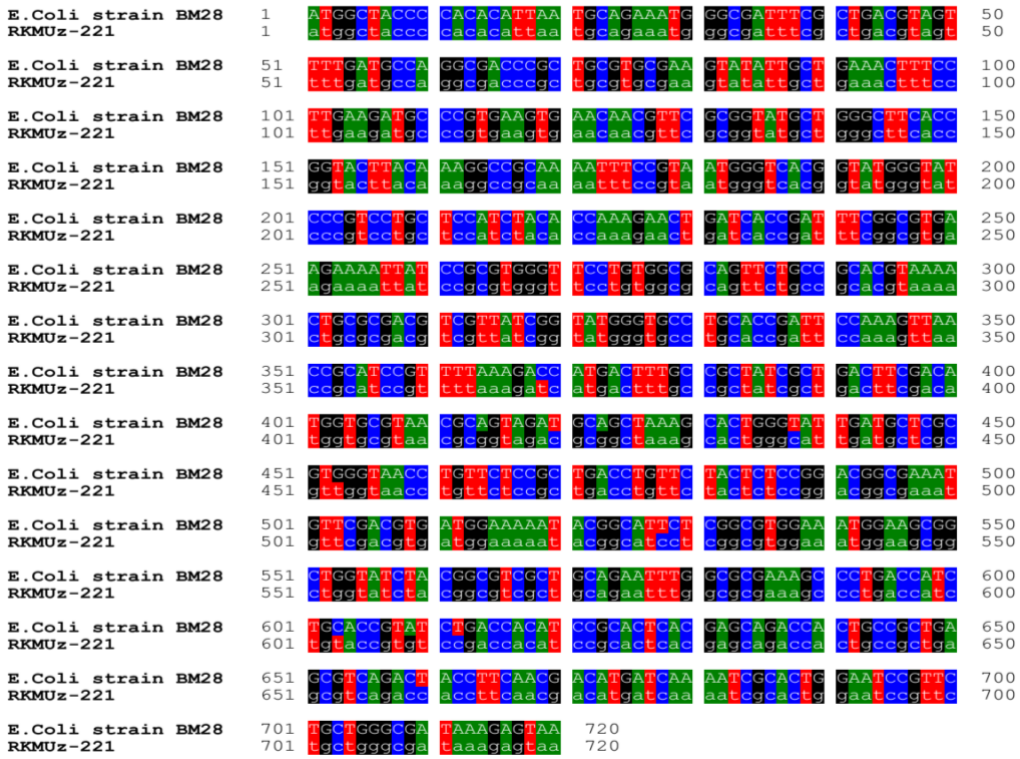


Figure 4. The alignment of the cloned *Ec*PNP sequence with the sequences of representative *E. coli* (BM28) from GenBank.

Expression and purification of *Ec*PNP

Fluorometric assay (ab204706, Abcam PLC, UK) was performed to study the expression level of the recombinant enzyme. The highest level of PNP expression (activity) was enriched at the 72 h of cultivation when adding 0.5% - 1.0% methanol to the medium every 24 h. The yeasts were homogenized as described in material and methods. Then homogenate was separated by centrifuging at 14.000 g for 30 min after gradient precipitation with 25%-65% ammonium sulphate. The fraction contains 15.8 mg/ml total proteins. The fraction containing PNP was analyzed with 10% SDS-PAGE electrophoresis by staining with Coomassie G-250 (Figure 5). Figure 5 shows the ≈ 28 kDa band which corresponds to the recombinant enzyme based on its nucleotide sequence.

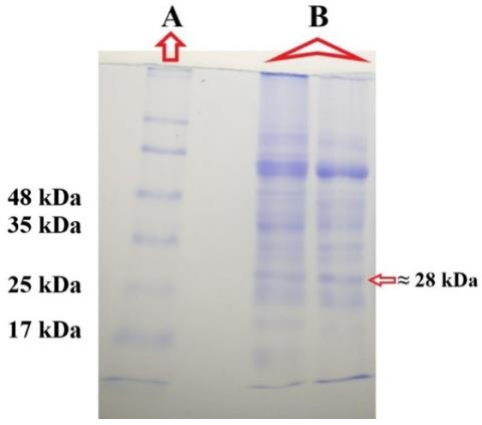


Figure 5. SDS-PAGE of fraction containing *rEc*PNP: (A) Tri-color restrained protein marker I (10-180kD), Bioland Scientific; (B) fraction after gradient precipitation with 25%-65% ammonium sulphate (3 and 5 µg protein per well).

Enzymatic hydrolysis of purine nucleosides by *Ec*PNP expressed in *Pichia pastoris*

The catalytic activity of the fractions contained *Ec*PNP was determined by hydrolysis of inosine (Figure 6). The level of hydrolysis and the identification of the formed hypoxanthine after hydrolysis were carried out by HPLC for 0-5 h.

As can be seen from the graph (Figure 7), the fraction containing recombinant *Ec*PNP leads to the hydrolysis of inosine within 5 h by more than 50% under our experimental conditions (Figure 8), which confirms its high hydrolytic activity.

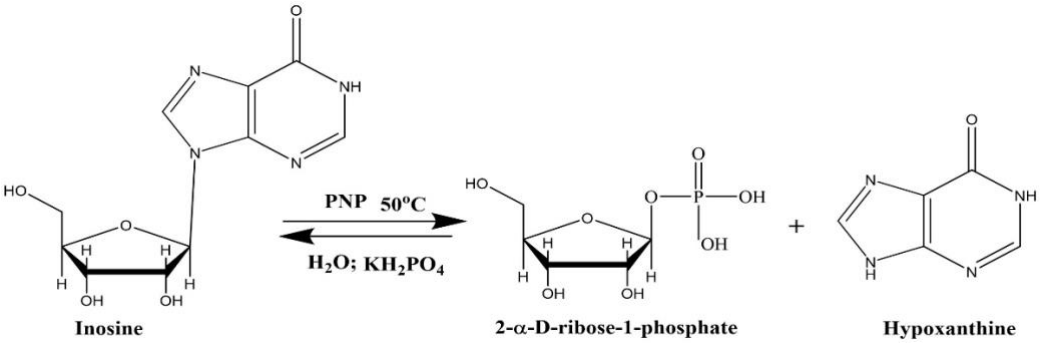


Figure 6. Enzymatic hydrolysis of Inosine (9-beta-D-ribofuranosylhypoxanthine) by purine nucleoside phosphorylase (PNP).

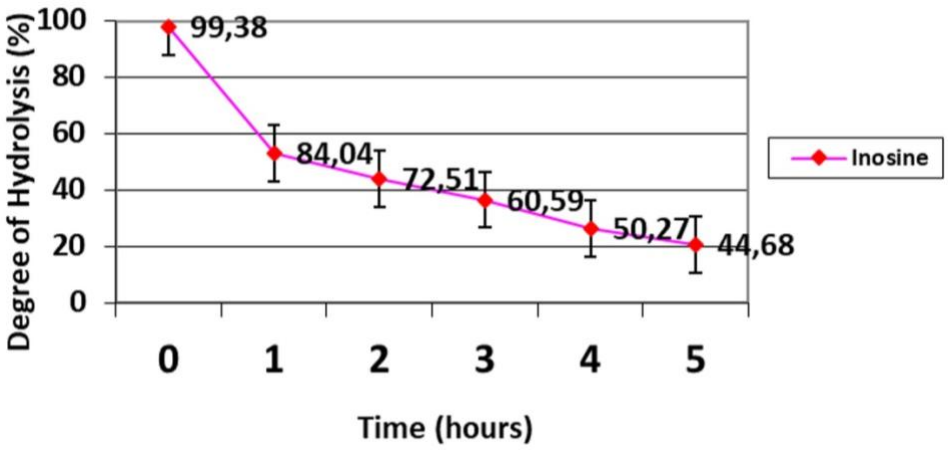
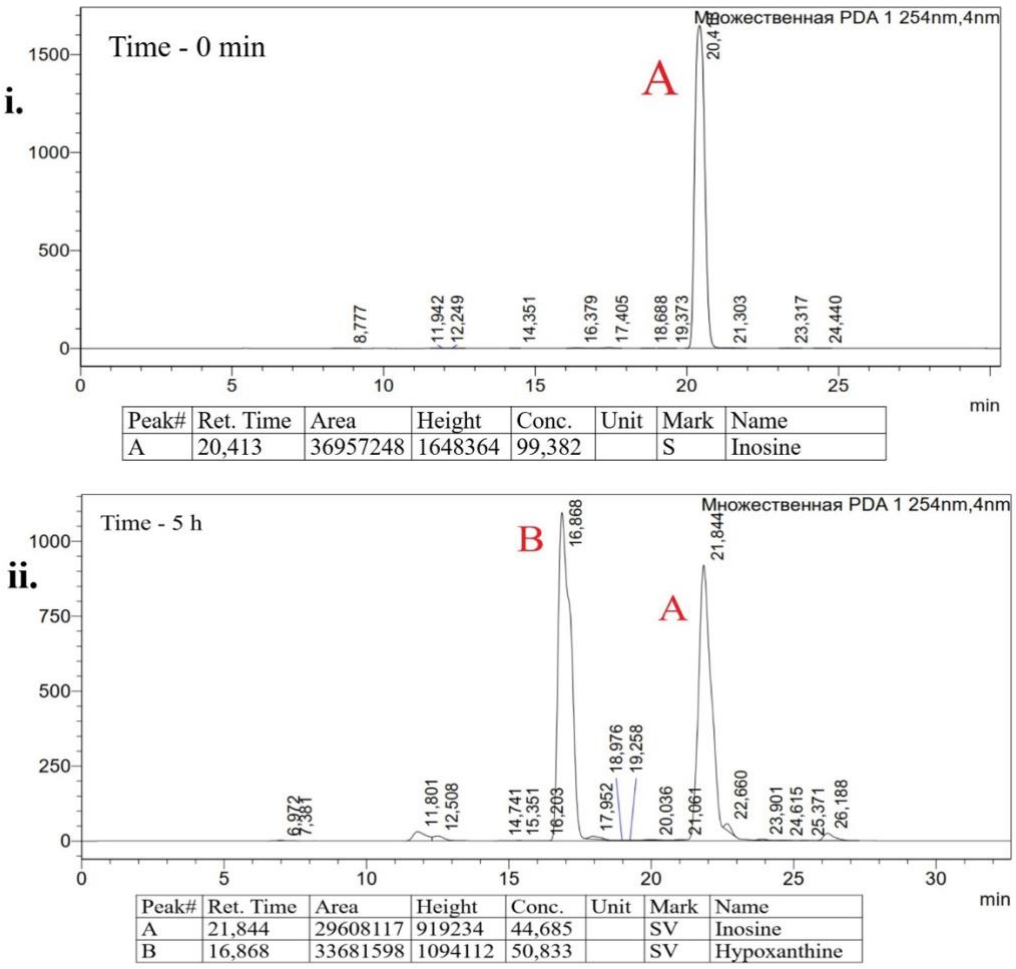


Figure 7. Time-course of enzymatic hydrolysis of Inosine catalyzed by recombinant *Ec*PNP expressed in *P. pastoris*. The graph shows the percentage of inosine hydrolyzed over a time period of 0 to 5 h.



Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
A	20,413	36957248	1648364	99,382		S	Inosine

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
A	21,844	29608117	919234	44,685		SV	Inosine
B	16,868	33681598	1094112	50,833		SV	Hypoxanthine

Figure 8. HPLC analysis of enzymatic hydrolysis of Inosine catalyzed by recombinant PNP. i-ii) The quantity of hydrolyzed Inosine in reaction mixture over time from 0 min to 5h.

DISCUSSION

The current study described the designing and cloning of the recombinant plasmid pPICZαA-PNP for the expression of the *Ec*PNP of conditionally pathogenic *E. coli* strain RKMUz-221 in *Pichia pastoris* yeast and enzymatic activity of recombinant *Ec*PNP. Besides, the study showed the *EcPNP* in the yeast for the first time.

Nowadays, *Pichia pastoris* is most effectively used in the large-scale production of heterologous recombinant proteins [12]. The successful creation of the recombinant pPICZαA-PNP plasmid is an important stage in the expression of the target protein in *Pichia pastoris* yeast. The obtained clones of pPICZαA-PNP after transformation into *E. coli* TOP10 were screened by PCR and restriction analysis, which confirmed the presence of the insert (the PNP-deoD gene) in the recombinant plasmid. The recombinant plasmid pPICZαA-PNP isolated from selected clones was then transformed into *Pichia pastoris* GS115 yeast for intracellular expression of protein. The selection of recombinant *Pichia clones* is carried out on a medium containing the antibiotic Zeocin™, which ensures the survival of only transformed yeasts [23]. The nucleotide sequencing of the *Ec*PNP gene of *E. coli* strain RKMUz – 221 showed 98.47% sequence similarity with *E. coli* strain BM28 [27] and eight different mutations in the PNP, represented as single nucleotide substitutions. It was interesting to see if these mutations would affect the structure and, most importantly, the functional activity of

the PNP enzyme, since some mutations may have no effect, while others may alter the enzymatic activity or stability of proteins. The identification of these mutations can provide important information for understanding the genetic diversity of PNP enzymes and their potential applications in biotechnology.

For the determination of influence of mutations in cDNA and enzymatic activity of the recombinant *EcPNP* in the stage of expression in *Pichia pastoris* cells used a fluorometric assay [26]. Fluorometric assay showed that the highest activity of expressed *Ec*PNP was enriched at 72 h of cultivation after induction with methanol (0.5% - 1.0%). The specific activity of recombinant *Ec*PNP was studied by hydrolysis of inosine to hypoxanthine and D-ribose because inosine as a purine type nucleoside is main substrate for purine nucleoside phosphorylase [28]. For this purpose, the recombinant *Ec*PNP containing fraction was separated from the yeast homogenate by gradient precipitation with 25%-65% ammonium sulphate. Because by using gradual changes in ammonium sulfate concentration, different proteins in a mixture can be precipitated sequentially based on their solubility properties [29-30]. In this protein purification stage using gradient ammonium sulfate precipitation, the optimal ammonium sulfate concentration needed for protein precipitation was determined based on the molecular weight of the recombinant protein. The salt concentration was titrated to find the value that resulted in maximum protein recovery while minimizing contamination from other protein impurities. The course of enzymatic hydrolysis of inosine by recombinant *Ec*PNP was analyzed using HPLC. The degree of hydrolysis of the inosine in reaction mixture was quantified by HPLC for 1-5 h. More than 50% of the inosine was hydrolyzed within 5 h under our experimental conditions, which indicates the high hydrolytic activity of the recombinant *Ec*PNP expressed in *Pichia*. It should be noted that the cell lysate of normal *Pichia pastoris* GS115 does not exhibit hydrolytic activity (HPLC chromatogram isn't provided). Thus, the revealed hydrolytic activity of recombinant *Ec*PNP indicates the ability to efficiently catalyze purine nucleosides, such as inosine, and can be used in the development of new antiviral or cytotoxic prodrugs based on modified nucleosides.

CONCLUSIONS

A new recombinant pPICZαA-PNP plasmid, consisting of 4256 base pairs, was successfully cloned to express the recombinant PNP of *E. coli* strain RKMUz-221 in the yeast *Pichia pastoris*. The molecular weight of the obtained recombinant *Ec*PNP, determined by electrophoresis was approximately 28 kDa. The enzymatic hydrolysis results revealed that the recombinant *Ec*PNP exhibited significant activity by hydrolyzing over 50% of inosine (9-beta-D-ribofuranosylhypoxanthine) within 5 hours. These findings demonstrate the potential of the obtained recombinant *Ec*PNP for generating modified nucleosides. Moreover, the expression of the recombinant enzyme in *Pichia pastoris* yeast provides a cost-effective and scalable approach for large-scale production.

ACKNOWLEDGEMENT

The study was carried out within the framework of project F-FA-2021-360, Ministry of Innovative Development of the Republic of Uzbekistan.

AUTHOR CONTRIBUTIONS

ShA and SS provided direction for this research idea; JA and SS: writing – review the manuscript, JA, ShKh, SG and SS: cloning, HPLC, data analysis; AM, AB: synthesis and purification of oligonucleotides (primers); KhN: DNA sequencing; OA, TS, and OY: cultivation of *Pichia pastoris* strains; FE and KhD: isolation of DNA, protein fractionation, SDS-PAGE. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

REFERENCES

[1] Berdis A. Nucleobase-modified nucleosides and nucleotides: Applications in biochemistry, synthetic biology, and drug discovery. Front Chem. 2022; 10:1051525.

[2] Pardi N, Weissman D. Nucleoside modified mRNA vaccines for infectious diseases. In: Kramps T, Elbers K (ed). RNA Vaccines: Methods and Protocols, Methods in Molecular Biology. Springer Science+Business Media: New York, 2017, pp 109-21.

[3] Cox RM, Wolf JD, et al. Therapeutically administered ribonucleoside analogue MK-4482/EIDD-2801 blocks SARS-CoV-2 transmission in ferrets. Nat Microbiol. 2021; 6: 11-18.

[4] Duffy K, Arangundy-Franklin S, et al. Modified nucleic acids: replication, evolution, and next-generation therapeutics. BMC Biol. 2020; 18: 112-27.

[5] Thiele I, Yehia H, et al. Production of Modified Nucleosides in a Continuous Enzyme Membrane Reactor. Int J Mol Sci. 2023; 24: 6081.

[6] Fateev IV, Kostromina MA, et al. Multi-Enzymatic Cascades in the Synthesis of Modified Nucleosides: Comparison of the Thermophilic and Mesophilic Pathways. Biomolecules. 2021; 11: 586.

[7] Fernández LJ. Multienzymatic synthesis of nucleic acid derivatives: A general perspective. Appl Microbiol Biotechnol. 2015; 99: 4615-27.

[8] Zhou X, Szeker K, et al. Recombinant purine nucleoside phosphorylases from thermophiles: Preparation, properties and activity towards purine and pyrimidine nucleosides. FEBS J. 2013; 280: 1475–90.

[9] Alexeev CS, Sivets GG, et al. Substrate specificity of E. coli uridine phosphorylase. Further evidences of high-syn conformation of the substrate in uridine phosphorolysis. Nucleosides Nucleotides Nucleic Acids. 2016; 0: 1-15.

[10] Bzowska A, Kulikowska E, et al. Purine nucleoside phosphorylases: Properties, functions, and clinical aspects. Pharmacol Ther. 2001; 88: 349–425.

[11] de Giuseppe PO, Martins NH, et al. Insights into phosphate cooperativity and influence of substrate modifications on binding and catalysis of hexameric purine nucleoside phosphorylases. PLoS One. 2012; 7: e44282.

[12] Ahmad M, Hirz M, et al. Protein expression in Pichia pastoris: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol. 2014; 98: 5301–17.

[13] Spohner SC, Quitmann H, et al. Expression of enzymes for the usage in food and feed industry with Pichia pastoris. J Biotechnol. 2015; 202: 118-34.

[14] Antonio MVG, Talita SC, et al. Comparison of yeasts as hosts for recombinant protein production. Microorganisms. 2018; 6: 38-62.

[15] Laurie V, Eric HF. A single protocol for extraction of gDNA from bacteria and yeast. Biotechniques. 2015; 58: 120-25.

[16] Purelink™ quick gel extraction kit. USA: Invitrogen; 2022.

[17] Sasmakov SA, Ashirov ON, et al. Obtaining of recombinant PreS2-S protein of surface antigen HBV in Pichia pastoris expression system. Vaccimonitor. 2021; 30(1): 27-32.

[18] T4 DNA Ligase. User Manual. USA: Invitrogen; 2022.

[19] Green RM, Sambrook J. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory Press: NY, USA, 2012. pp. 177

[20] Green RM, Sambrook J. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory Press: NY, USA, 2012. pp. 94-99.

[21] Michael N, Thomas MD. DNA sequencing with the SeqStudio. McGill: Montreal, Canada, 2020.

[22] DNA Sequencing Clean-Up Kit. USA: Zymo Research; 2022.

[23] EasySelect™ Pichia Expression Kit. USA: Invitrogen; 2022.

[24] He F. Laemmli-SDS-PAGE. Bio Protoc. 2011; 1: e80.

[25] Waterborg JH, Matthews HR. The Lowry Method for Protein Quantitation. Methods Mol Biol. 1994; 32: 1-4.
[26] Purine Nucleoside Phosphorylase Activity Assay Kit. UK: Abcam; 2022.
[27] NCBI (2022). Reference Sequence: NZ_CP102380.1. https://www.ncbi.nlm.nih.gov/assembly/GCF_025563515.1#/def
[28] Gaofei L, Tiantong Ch, et al. Efficient synthesis of purine nucleoside analogs by a new trimeric purine nucleoside phosphorylase from Aneurinibacillus migulanus AM007. Molecules. 2020; 25(1): 100-13.
[29] Pringels L, Broeckx V, et al. Abundant plasma protein depletion using ammonium sulfate precipitation and Protein an affinity chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. 2018; 1089: 43-59.
[30] Wingfield P. Protein precipitation using ammonium sulfate. Curr Protoc Protein Sci. 2001; A3: A3F-8F.