Current Trends in Microbiology
Vol. 12, 2018

Original Article

Protein pattern and transglycosylation activity of *Geobacillus kaustophilus*

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ABSTRACT

Thermophile bacterium *Geobacillus kaustophilus* was able to synthesize 5-fluoro-2´-deoxyuridine (floxuridine) to 52 $mg.L^{-1}.h^{-1}$ using whole cells. The presence of 1 $mmol.L^{-1}$ $ZnCl_2$ enhanced transglycosylation reaction. The reaction was also enhanced when 2-deoxyuridine (2'dur) was, even partially, replaced with 5-fluoro-2´-deoxyriboside (5'Fdri). The highest production of floxuridine was observed at the 5'Fdri to 2'dur ratio of 5:1, i.e. when 5'Fdri was in great excess. *G. kaustophilus* can accept both 6-oxo- and 6-aminopurine nucleotides as substrates, but oxo-nucleotides are more efficient substrates than 2´-amino modified compounds. Mass spectrometric analyses resulted in the identification of 23 proteins representing metabolic enzymes, and purine nucleotide phosphorylase DeoD subunit. This strain can be used as a catalyst in the biosynthesis of 2´-fluorinate nucleotides.

KEYWORDS: transglycosylation, floxuridine, *Geobacillus kaustophilus.*

INTRODUCTION

Thermophilic bacteria are a source of extremely stable enzymes and are attractive for industrial bioprocesses [1]. Among other topics, the attention of the industrial sector is focused on the potential of thermostable purine polynucleotide phosphorylases as biocatalysts in the synthesis of modified nucleosides [2]. Nucleotide phosphorylases (NPs) are divided into two groups, based on their substrate specificity: purine NPs (PNPs, E.C. 2.4.2.1) and pyrimidine NPs (PyNPs, E.C. 2.4.2.2). The PNPs from various sources form a broad spectrum of N-ribohydrolases and transferases. Some of them were isolated and obtained in a crystalline state and their structures were described, which gave the basis for PNPs' classification [3]. The first class is represented by low-molecular-mass homotrimers (80-100 kDa) that specifically catalyze 6-oxopurines and their nucleosides ("Ino-Guo phosphorylases"). These enzymes were isolated from mammalian tissues [4], from spores and vegetative cells of *Bacillus cereus* [5], and from *Bacillus stearothermophilus* THG-2 [6]. The second class is represented by high-molecular-mass homohexamers (110-160 kDa) that catalyze 6-oxo- and 6-aminopurines. This class of enzymes can be produced by *Escherichia coli*, *Salmonella typhimurium* [7], *Klepsiella* [8], and *Sulfolobus solfataricus* [9]. One of the PNPs from *Escherichia coli*, the so-called "Xao phosphorylase", differs from other hexameric PNPs by its inability to accept Ado as a substrate, while it excellently accepts Xao, a 6-oxopurine nucleoside [3].

In the presence of inorganic phosphate, PNPs cleave the glycosidic bond of ribo- and deoxyribonucleosides to give a purine base and (deoxy)ribose-1-phosphate. Besides the natural substrates such as thymidine and uridine, their analogues with fluoro-modified sugars were also used for the study of phosphorolysis. These substrates can be used as pentofuranosyl donors in the

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enzymatic transglycosylation of pharmacologically valuable 2´-fluorosubstituted purine nucleotides [2]. 6-halogenated purine nucleotides and their derivatives seem to be promising antiviral and anticancer agents [10]. For example, 6-chloropurine-2´-deoxyriboside and 6-chloropurine riboside are active against hepatitis C virus and SARS coronavirus [11]. Human NPs are therapeutic targets in tumoral cells; they are treated with inhibitors that make DNA replication impossible by limiting the available pool of nucleotides [1]. Another strategy is to administrate nucleobase analogues, which are harmless when conjugated with a pentose but cytotoxic after the phosphorolysis by NPs [12, 13]. Chemical synthesis of nucleoside analogues requires multiple reaction steps, usually followed by a more-or-less complicated purification [10]. However, it has been reported that a group of lactic acid bacteria (*Lactobacillus helveticus*, *L. leischmannii*, *L. reuteri*, *L. lactis and L. animalis*) contains 2-N-deoxyribosyltransferases, which catalyze transglycosylation between purine or pyrimidine bases and nucleosides in a one-step reaction. *L. animalis* was used for biotransformation of floxuridine (5-fluorouracil 2´-deoxyriboside), which is active against colorectal, pancreatic, breast and neck cancers [14].

Different conditions were tested to optimize the reaction parameters for obtaining 6-halogenated purine nucleotides. The presence of phosphate in the reaction for nucleotide phosphorolysis is essential [15] and optimum phosphate concentration is between 30-40 mM at pH 7.0. Transglycosylation reactions are reversible [16]. An excess of substrates improves transglycosylation [10]. Biosynthesis of 6-chloropurine-2-deoxyriboside (6-ChP2Deo) in the presence of an excess of 6-halogenated base or an excess of nucleoside donors led to a lower yield of 6-ChP2Deo. At the ratio 6:2 mmol.L^{-1} (6-chloropurine/D-uridine), the production of 6-ChP2Deo increased significantly.

The goal of our search was to characterize the proteins and transglycosylation activity of *Geobacillus kaustophilus*. The substrate specificity of the cells was relatively broad and natural substrates were significantly efficient in transglycosylation reactions.

MATERIALS AND METHODS

Growth and cultivation

Geobacillus kaustophilus HTA426 was grown in a broth containing 4 g.L^{-1} yeast extract, 8 g.L^{-1} peptone and 2 g.L^{-1} NaCl, at pH 7.2, for 48 h at 58 °C. Cells were harvested by centrifugation at 8000 xg for 10 min, at 4 °C. The cell suspension was washed with a standard buffer (10 mmol.L^{-1} Tris-HCl pH 7.6, 10 mmol.L^{-1} $MgCl_2$, 40 mmol.L^{-1} NH_4Cl, 6 mmol.L^{-1} 2-mercaptoethanol) and frozen.

Isolation of proteins for 2-D electrophoresis

Cells were suspended in 0.7 mL of standard buffer with 0.2 mmol.L^{-1} phenylmethylsulfonyl fluoride (PMSF) and disrupted with glass beads in a FastPrep cell disruptor FP 120 5x 30 sec, at 4 °C. Glass beads and insoluble materials were removed by centrifugation for 10 min at 10000 xg. The supernatant solution was centrifuged at 14000 xg for 20 min and the sediment was removed. The supernatant was incubated with benzoase as recommended (Sigma). Proteins were concentrated by precipitation with CCl_3COOH (trichloroacetic acid (TCA)) to a final concentration of 5%. After 5 min of incubation on ice, the proteins were recovered by centrifugation at 14000 xg for 20 min, at 4 °C. The sediment was washed with cold acetone and ether and suspended in 5 µL deionized water and solubilized in a solution containing 1.5 g ultrapure urea, 0.1 g 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma), 40 mg dithiothreitol (DTT), 160 µL Ampholine pH 4.6 (LKB) and 40 µL Pharmalyte pH 5.8 (Pharmacia) (total volume of 2.5 mL).

Electrophoresis

Preparative electrophoretic separation (isoelectric focusation (IEF)-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)) was performed using the Bio-Rad equipment (Hercules, CA, USA). First-dimensional gels (3 x 200 mm) were run for 16 h at 1000 V. The separation in the second dimension was carried out in 12.5% SDS-polyacrylamide gel (200 x 200 mm) at 250 V. Gel was stained with colloidal Coomassie Brilliant Blue G250 (CBB).

Enzymatic in-gel digestion

CBB-stained protein spots were excised from the gel, cut into small pieces, and washed several times with a solution containing 10 mmol.L^{-1} dithiothreitol and 0.1 mol.L^{-1} 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). After complete distaining, the gel was reconstituted in a cleavage buffer containing 0.01% 2-mercaptoethanol, 0.1 mol.L^{-1} N-ethylmorpholine acetate, 1 mmol.L^{-1}

$CaCl_2$, 10% MeCN and sequencing grade trypsin (50 ng.μL^{-1}, Promega). The resulting peptides were extracted to 40% MeCN/0.5% trifluoroacetic acid (TFA). The samples were purified and concentrated using C18 ZipTips^{TM} Millipore prior to mass spectrometric analysis.

MALDI-mass spectrometry

A solution of α-cyano-4-hydroxycinamic acid (Sigma) in 50% MeCN/0.2% TFA was used as the matrix. Mass spectra were measured on the Bruker BIFLEX II equipped with SCOUT 26 sample inlet and a nitrogen laser 337 nm (Laser Science, USA).

Detection of transglycosylation activity

The detection was performed essentially as described in [17]. Cells were centrifuged at 10000 xg for 10 min and the sediment was washed with a standard buffer and used as the source of enzymatic activity. The sediment from 2 mL of the culture was suspended in 50 μL of the solution containing 10 mmol.L^{-1} base and 30 mmol.L^{-1} starting nucleoside in 30 mmol.L^{-1} K-phosphate buffer, pH 7.2. The reaction was performed at 55 °C while the mixture was being shaken at 300 rpm for 48 h. Samples of 1 μL were taken and analyzed by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). TLC was performed on silica gel plates using chloroform/methanol (85:15 v/v) as a mobile phase. Samples of 5 μL were applied on the plate and spot intensity was compared with the standard solution of known concentration. HPLC analysis was carried out with a C-18 column (200 x 4 mm) and the detection was performed at 254 nm. The separation was carried out at room temperature with the gradient of water/acetonitrile or water/methanol at 1 mL.min^{-1}.

RESULTS AND DISCUSSION

Proteins were extracted from 24-h-old cultures and analyzed by means of two-dimensional electrophoresis, and visualized using Coomassie Brilliant Blue (Fig. 1). To eliminate a possible proteolytic activity of the bacterial cell extract, in a parallel experiment, [^{35}S]-labelled protein extract was incubated in the presence of PMSF at room

Fig. 1. 2D map of *Geobacillus kaustophilus* proteins from 24-h-old cultures. The proteins were separated on 3-10 linear IPG strips, followed by electrophoresis in 12.5% SDS-polyacrylamide gel. The selected CBB-stained proteins (designated from 1 to 23) were in-gel digested as described in [23].

temperature for 30 min and the radioactivity of the TCA precipitate that was retained on the membrane filter was measured. No valuable loss of radioactivity was observed. The 23 differential spots were subjected to further analysis and the proteins were identified using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) on the basis of peptide mass matching. To obtain an overview of the metabolic activity, protein spots representing glycolytic enzymes, citric acid cycle, metabolism of amino acids, nucleic acid and protein synthesis factors were detected (Table 1). The presence of lipoamide dehydrogenase E1 (spot 16) was found. It is an important part of the pyruvate dehydrogenase complex; the cells quickly activate it as soon as they encounter oxygen, and it supplies acetyl-CoA for anaerobic reactions. The citrate acid cycle is the major site of carbon chain oxidation into CO_2 and water, *via* Ac-CoA and several amino acids, in a manner that conserves the liberated free energy for utilization in ATP synthesis. Acetyl-CoA acetyltransferase was detected in two abundant spots (10 and 11). This enzyme participates in the following metabolic processes: fatty acid metabolism, valine, leucine, isoleucine and lysine degradation, tryptophan metabolism, pyruvate metabolism, benzoate degradation *via* CoA-ligation, propanoate metabolism, butanoate metabolism, and two-component system. Nicotinamide adenine dinucleotide phosphate **(**NADPH) dehydrogenase (spot 14) catalyzes the reduction of the double bonds of alpha and beta-unsaturated aldehydes and ketones. ATP synthase (EC 3.6.3.14) is an important enzyme that creates an energy storage in the form of adenosine triphosphate (ATP). ATP is the most commonly used "energy currency" of cells for most organisms. Large-enough quantities of ATP create a transmembrane proton gradient, which is used by fermenting bacteria that do not have an electron transport chain. These bacteria rather hydrolyze ATP to make a proton gradient, which they use to drive flagella and to transport nutrients into the cell. In respiring bacteria under physiological conditions, ATP synthase, in general, runs in the opposite direction, creating ATP while using the proton motive force created by the electron transport chain as a source of energy. Subunits of ATP synthase alpha (spot 3) and beta (spot 5) are found in the flagellar ATPase and the termination factor Rho. Flagellin protein (spot 17) is the principle substituent of a bacterial flagellum, and is present in large amounts on nearly all flagellated bacteria. The protein synthesis elongation factor Tu (spot 8) is a more abundant protein in comparison with EF-Ts (spot 15) and EF-P (spot 23). Superoxide dismutase (spot 20), within cells, forms the superoxide that inactivates many enzymes, including aconitase, and releases potentially toxic iron from its storage proteins. Flavin mononucleotide (FMN)-dependent NADH azoreductase (AzrG, spot 19) exhibits a wide-range of degrading activity towards several azo dyes. *Geobacillus* was found promising for sustainable utilization as an azo-degrading strain for AzrG carrying whole-cell wastewater treatments for azo pollutants under high temperature conditions [18]. In previous studies, an orphan lantibiotic precursor homologous to LanA lantibiotic gene cluster of *G. denitrificans* was detected [19]. In *G. kaustophilus*, we detected a subunit of purine nucleotide phosphorylase *deoD* gene (spot 18). This finding will lead to the analysis of the substrate specificity of the bacterium, including substrates such as 6-amino purines, their nucleotides and analogues.

Synthesis of adenosine from uridine

The cells of *G. kaustophilus* were harvested at different times of cultivation and the maximum adenosine synthesis was achieved at the end of the exponential phase of growth and remained constant from 6 to 12 h (Fig. 2). All experiments were carried out with the same amount of cells (5×10^{-6} cells) at a temperature of 55 °C. The effect of temperature on the activity was examined in the range from 30 to 90 °C. Maximum adenosine synthesis was obtained from 60 to 70 °C, which is higher than the optimal growth temperature. At temperatures higher than 70 °C, the activity declined (Fig. 3).

Biosynthesis of 5-fluorouracil-2-deoxyriboside (floxuridine)

The cells of *G. kaustophilus* were incubated in the medium containing yeast extract, NaCl, and glucose, at pH 7.2 and temperature 50 °C. In various stages of growth, the cells were collected and suspended in 30 $mmol.L^{-1}$ K-phosphate buffer at the concentration of 10^{10} colony-forming units (CFUs). 1-mL aliquots of the cells were mixed with 2 $mmol.L^{-1}$ 5-fluoro-2-deoxyriboside (5´Fdri) and

Table 1. Identification of selected proteins of thermophilic bacterium *G. kaustophilus*.

Protein	MW [kDa]	pI	Peptides	SC [%]	Spot No.
Acetyl CoA dehydrogenase OS = *Geobacillus kaustophilus* (strain HTA426) GN = GA 30006	65.8	5.5	11	41.2	1
Pyruvate kinase OS = *Geobacillus kaustophilus* (strain HTA426) GN = GK2739	62.3	5.7	9	12.8	2
ATP synthase subunit alpha OS = *Geobacillus kaustophilus* (strain HTA426) GN = atpA	54.8	5.4	2	3.2	3
Glutamine synthetase OS = *Geobacillus kaustophilus* (strain HTA426) GN = glnA	50.5	5.1	16	40.3	4
ATP synthase subunit beta OS = *Geobacillus kaustophilus* (strain HTA426) GN = atpD 1	51.8	4.9	19	42.3	5
Malic enzyme OS = *Geobacillus kaustophilus* (strain HTA426) GN = GK1440	51.5	5.5	11	25.1	6
Isocitrate lyase OS = *Geobacillus kaustophilus* (strain HTA426) GN = GK0676	47.3	5.4	9	31.8	7
Elongation factor Tu OS = *Geobacillus kaustophilus* (strain HTA426) GN = tuf	43.3	4.7	9	34.4	8
Histidinol dehydrogenase OS = *Geobacillus kaustophilus* (strain HTA426) GN = hisD	46.0	5.3	5	9.2	9
Peptidase T OS = *Geobacillus kaustophilus* (strain HTA426) GN = pepT	45.7	5.1	13	38.0	10
Acetyl-CoA acetyltransferase OS = *Geobacillus kaustophilus* (strain HTA426) GN = GK3397	40.8	5.6	15	30.1	11
Acetyl-CoA acetyltransferase OS = *Geobacillus kaustophilus* (strain HTA426) GN = GK2035	43.2	5.8	16	29.5	12
Succinyl-CoA ligase ADP-forming subunit beta OS = *Geobacillus kaustophilus* GN = suc CP	41.8	5.1	8	26.9	13
NADPH dehydrogenase OS = *Geobacillus kaustophilus* (strain HTA426) GN = namA	37.7	6.1	25	0.6	14
Elongation factor Ts OS = *Geobacillus kaustophilus* (strain HTA426) GN = tsf	32.6	5.3	4	14.3	15
Dehydrogenase E1 component β subunit (Lipoamide) OS = GN´= GK 1059	35.4	5.0	19	61.2	16
Flagelin protein OS = *Geobacillus kaustophilus* (HTA426) GN = hag	31.9	5.1	16	35.0	17
Purine nucleoside phosphorylase DeoD-type OS = *Geobacillus kaustophilus* GN = deoD	25.8	5.1	7	41.1	18
FMN-dependent NADH-azoreductase OS = *Geobacillus kaustophilus* (strain HTA426) GN = azoR	23.7	5.6	7	40.8	19
Superoxide dismutase OS = *Geobacillus kaustophilus* (strain HTA426) GN = GK2457	22.9	5.5	2	25.0	20
Hypoxanthine-guanine phosphoribosyltransferase OS = *Geobacillus kaustophilus* GN = GK0061	21.6	5.0	3	17.1	21
Peroxiredoxin OS = *Geobacillus kaustophilus* (HTA426) GN = GK 2575	21.0	5.0	9	45.5	22
Elongation factor P OS = *Geobacillus kaustophilus* (strain HTA426) GN = efp	20.8	5.3	4	36.8	23

Note: Digested proteins were extracted to 40% MeCN/0.5% TFA, purified and concentrated using C18 ZipTips^{TM} Millipore prior to MALDI-MS (Abbreviations used: OS = organism name, GN = gene name, MW = molecular weight, pI = isoelectric point, SC = sequence coverage.).

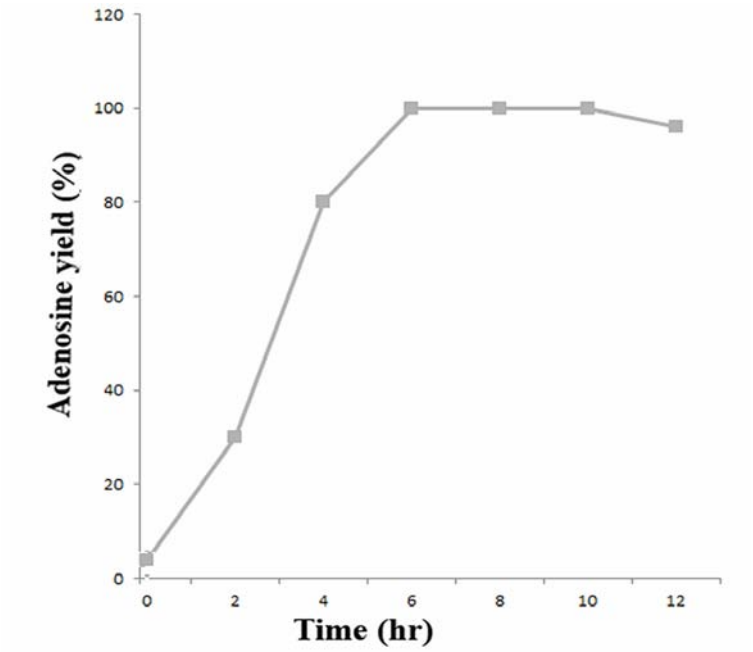

Fig. 2. Synthesis of adenosine from uridine. The effect of growth phase on adenosine synthesis was always examined with the same amount of cells (5×10^{-6}) and at the temperature of 55 °C. The maximum synthetic activity was found in the cells from the stationary phase.

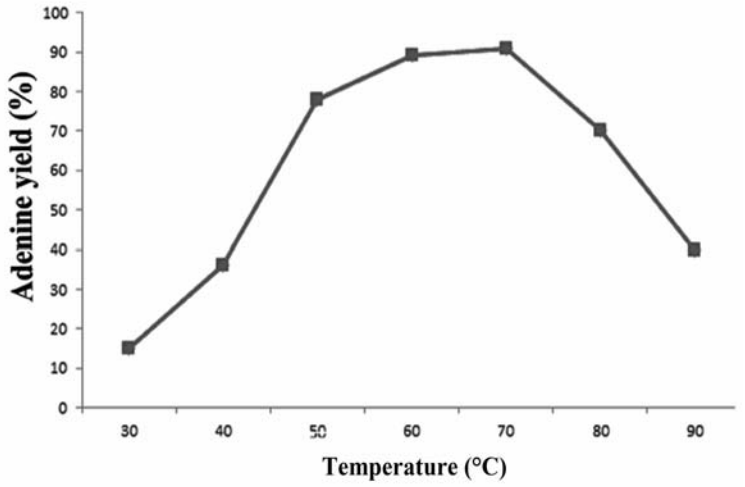

Fig. 3. Effect of temperature on synthesis of adenosine from uridine. The cells from a 12-h cultivation (5×10^{-6}) were incubated at different temperatures and adenosine yield was examined. Maximum activity was found between 60-70 °C; at the higher temperatures the activity decreased.

6 mmol.L^{-1} 2´-deoxyuridine (2´dur). The reactions were performed at 50 °C. After a 2-hr cultivation, samples (300 mL) were taken in triplicates, and the cells were removed by centrifugation. The supernatant was analyzed by TLC or HPLC. Reaction product was compared with the reference standard or it was analyzed using MS-HPLC. Maximum yield of floxuridine was achieved with the cultures at the end of the exponential phase of growth (Fig. 4). The reactions were realized with different cations and concentrations (from 1 mmol.L^{-1} to 15 mmol.L^{-1}).

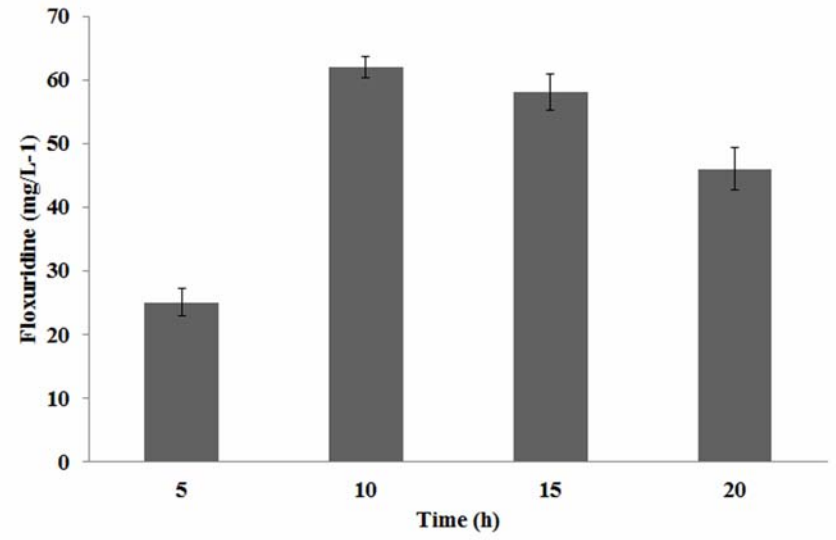

Fig. 4. Synthesis of floxuridine from 5-fluoro-2-deoxyriboside and 2´-deoxyuridine. After the cultivation, the cells were removed by centrifugation and the supernatant solution was analyzed by HPLC chromatography. The maximum production of floxuridine was achieved in the cultures from the end of the exponential phase.

When the cells were incubated in the presence of 1 mmol.L^{-1} $ZnCl_2$, the production of floxuridine was increased from 30 mg.L^{-1}.h^{-1} (the result of control experiments without added cations) to 52 mg.L^{-1}.h^{-1}. No significant changes in the production of floxuridine were observed in the presence of 1 or 5 mmol.L^{-1} concentrations of Mg^{2+}, Ca^{2+}, Fe^{2+}, or Co^{2+}. Glycosidic bond cleavage in purines and pyrimidines by the phosphorolysis mechanisms catalyzed by PNPs and PyNPs is a critical process in the metabolism of nucleosides [20]. One of the important features of NP-catalyzed reactions is that they are reversible; NPs can catalyze both the phosphorolysis of a nucleotide, and its synthesis from an acceptor nucleobase and a pentose-1-phosphate [1].

We found that changes in the ratio of 5´Fdri/2´dur affected the production of floxuridine. Significantly higher activity (about 40 %) was observed with an excess of 5´Fdri (5:1). These data indicate that the excess of a substrate may improve transglycosylation reaction.

There are two groups of PNPs: one has a substrate specificity for 6-oxopurine nucleotides and the other for 6-oxo- and 6-aminopurine nucleotides. The data (Table 2) show that *G. kaustophilus* can accept both 6-oxo- and 6-aminopurine nucleotides as substrates, but natural purine nucleotides are superior substrates to 2´-amino-modified compounds.

Table 2. Substrate specificity of *Geobacillus kaustophilus* PNPs.

Substrates	**V_{max} [U.mg^{-1}]**
adenosine	120
inosine	78
D-adenosine	118
2´-amino-2-deoxyadenosine	2.2
2´-amino-2´-deoxyinosine	2.8

Note: Activities were determined at 50 °C.

The thermostable PNPs were used in the synthesis of pharmacologically active components [21].

CONCLUSION

Enzymes from mesophilic bacteria exhibit a limited ability to withstand extreme conditions such as low or high pH, the presence of organic solvents or higher temperature. Namely, temperature is an important factor in biotechnological processes, because higher temperatures usually lead to a lower viscosity of media and better solubility of substrates, which is desirable [22]. Thermophilic bacteria are more stable under extreme conditions and this is an advantage during the isolation of their enzymes in active forms.

Our data indicate that *G. kaustophilus* can be used as a catalyst in the transglycosylation reactions with adenosine or D-adenosine during the synthesis of 2´-fluorinated adenine nucleosides. Purine nucleotides with a modified C´2-carbon atom of pentafuranose ring are an important part of oligonucleotides with a potential application in cancer chemotherapy.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ABBREVIATIONS

2´dur	:	2´-deoxyuridine
5´Fdri	:	5-fluoro-2-deoxyriboside
6-ChP2Deo	:	6-chloropurine-2-deoxyriboside
ATP	:	adenosine triphosphate
AzrG	:	FMN-dependent NADH azoreductase
CBB	:	Coomassie Brilliant Blue (G250)
CFU	:	colony-forming unit
CHAPS	:	3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate
DTT	:	dithiothreitol
FMN	:	flavin mononucleotide (or riboflavin-5′-phosphate)
GN	:	gene name
HPLC	:	high-performance liquid chromatography
IEF	:	isoelectric focusation
MALDI-MS	:	matrix-assisted laser desorption/ionization mass spectrometry
MeCN	:	acetonitrile
MW	:	molecular weight
NADPH	:	nicotinamide adenine dinucleotide phosphate (a reduced form)
NPs	:	nucleotide phosphorylases
OS	:	organism name
pI	:	isoelectric point
PMSF	:	phenylmethylsulfonyl fluoride
PNPs	:	purine nucleotide phosphorylases
PyNPs	:	pyrimidine nucleotide phosphorylases
SC	:	sequence coverage
SDS-PAGE	:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	:	trichloroacetic acid
TFA	:	trifluoroacetic acid
TLC	:	thin layer chromatography

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