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# Optimization of cold-adapted alpha-galactosidase expression in *Escherichia coli*

V.A. Golotin [a, b, *], L.A. Balabanova [a, b], Yu. A. Noskova [a], L.V. Slepchenko [a], I. Yu Bakunina [a], N.S. Vorobieva [b], N.A. Terenteva [a], V.A. Rasskazov [a]

[a] G.B. Elyakov Pacific Institute of Bioorganic Chemistry FEB RAS, Vladivostok, Russia
[b] Far-Eastern Federal University, Vladivostok, Russia

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ABSTRACT

α-Galactosidase (α-PsGal) of the cold-adapted marine bacterium *Pseudoalteromonas* sp. KMM 701 was cloned into the pET-40b(+) vector to study its properties and to develop an effective method for modifying human B-erythrocytes into O-blood group. The use of heat-shock as a pre-induction treatment, IPTG concentration of 0.2 mM and post-induction cultivation at 18 °C for 20 h in the developed MX-medium allowed increasing the recombinant *Escherichia coli* Rosetta (DE3)/40Gal strain productivity up to 30 times and the total soluble α-PsGal yield up to 40 times.

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## 1. Introduction

α-Galactosidases (α-D-galactoside galactohydrolase; EC 3.2.1.22) catalyze hydrolysis of terminal α-galactose residues in oligosaccharides and polymeric galactomannans. Some galactosidases described in the literature have a marine origin. The main interest in marine α-galactosidases that involved in the transformation and utilization of carbohydrates and carbohydrate-containing biopolymers is determined by their catalytic advantages over its terrestrial counterparts. Their unique properties, specificity, physiological pH and temperature for the manifestation of maximum activity have been found in a number of potential applications in biotechnology and medicine [1–3]. α-Galactosidases are widespread in the marine bacteria [4,5]. α-Galactosidase from the marine bacterium *Pseudoalteromonas* sp. KMM 701 is the only fully-characterized among the enzymes of marine origin to date [6,7]. The marine bacterium *Pseudoalteromonas* sp. KMM 701 α-galactosidase has been found to belong to GH36 family, according to its amino acids sequence in CAZy classification. In addition, the α-galactosidase has rare specificity. It is able to inactivate the serological activity of human B-erythrocytes converting it into the "universal donor" blood of group O [7, 8.]. The recombinant version of the enzyme demonstrated the more efficient conversion of the human red blood cells compared to the suggested green coffee bean α-galactosidase [8,9].

The enzyme has a dimer molecular weight of 160 kDa, is stable at 20 °C for 24 h and has pH optimum for catalysis within the range of 6.7–7.7 [6]. The recombinant analog of the enzyme was previously obtained [10].

However, it remains a problem to obtain sufficient amounts of the recombinant cold-adapted α-galactosidase of a large molecular weight (160 kDa) for an industrial scale usage. The protein has hydrophobic molecule surface facilitating aggregation during post-translation modification and protein accumulation in the cells. Another significant restriction for the production of recombinant protein is its accumulation in inclusion bodies as an insoluble protein fraction [11].

The main goal of this work was to optimize α-PsGal expression

*Abbreviations:* α-PsGal, α-Galactosidase from the cold-adapted marine bacterium Pseudoalteromonas sp. KMM 701; KMM, Collection of Marine Microorganisms; CAZy, Carbohydrate-Active enZYmes Database; LB, Luria-Bertani medium; TB, Terrific Broth medium; LBBM, buffered Luria-Bertani medium with magnesium; LBBNM, buffered Luria-Bertani medium with magnesium and extra NaCl; LBBMG, buffered Luria-Bertani medium with magnesium and glycerol; LBBSMG, buffered Luria-Bertani medium with magnesium, glycerol and sorbitol; MX, Medium for eXpression; IPTG, isopropyl β-D-1-thiogalactopyranoside; OD, Optical Density; 4-NGP, 4-nitrophenyl α-D-galactopyranoside; ε, molar extinction coefficient.

* Corresponding author. Elyakov Pacific Institute of Bioorganic Chemistry, Prospect 100 let Vladivostoku 159, 690022 Vladivostok, Russia.
*E-mail address:* golotin@bk.ru (V.A. Golotin).

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and processing in the *E. coli* cells through adjusting the cultivation and induction conditions of the recombinant strain *E. coli* strain.

## 2. Materials and methods

### 2.1. Recombinant α-Galactosidase plasmid construction

The α-PsGal gene was amplified with the use of Encyclo Taq DNA polymerase (Evrogen), genomic DNA of the marine bacterium *Pseudoalteromonas* sp. KMM 701 (Collection of Marine Microorganisms, PIBOC FEB RUS, http://www.piboc.dvo.ru) and the gene-specific forward and reverse primers containing NcoI and SalI restriction sites, respectively (forward: 5′- ATTACCATGGCCGACACTAAATCATTTTATCGATTAGACA -3′; reverse: 5′-ACACGTCGACTTACGCTTTGTTGAGCTCAAATATAAGC-3′). PCR was performed in automatic amplifier (Eppendorf) as the following scheme: 95 °C - 3 min, (95 °C—15 s, 72 °C—2 min) x 38 cycles. Restricted PCR-product was ligated into the pET-40b(+) vector (Novagen). Restriction endonucleases and T4 DNA ligase were purchased from "Thermo Scientific". The resultant plasmid was sequenced using the automated PE/ABI 310 DNA sequencer and the PE/ABI-ABI PRISM BigDye Terminator cycle sequencing Ready Reaction Kit v.3.1 (Applied Biosystems). Preparation of *E. coli* competent cells and transformation were performed according to the standard methods [12].

### 2.2. Cultivation of E. coli cells

For all experiments on α-PsGal expression optimization, the obtained recombinant plasmid was transformed into the *E. coli* Rosetta(DE3) cells by the standard heat-shock method [12]. The recombinant cells were grown on LB plate (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar per 1 L, pH 7.5) containing 25 μg/mL of kanamycin overnight at 37 °C. A single colony was picked and grown at 200 rpm in LB with 25 μg/mL of kanamycin at 37 °C for 12 h. A 1 ml of overnight culture was used for the recombinant α-PsGal expression for one glass flask with 50 ml medium at the different cultivating conditions.

### 2.3. Determining optimal temperature and inducer concentration

Overnight culture was 50-times diluted in several 250 ml-glass flask with 50 ml of a fresh LB containing 25 μg/mL of kanamycin. The culture was cultivated at 37 °C until the cell density was reached an $OD_{600}$ of 0.6—0.8 and then was cooled to room temperature. The different concentrations of the expression inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) from 0.1 to 0.5 mM was added to culture, and cultivation was continued at different temperatures from 12 to 24 °C.

### 2.4. Determining optimal medium composition

On the base of the standard LB medium for *E. coli* cultivation, the following modified media used for the expression:

- TB (12 g bacto-tryptone, 24 g yeast extract, 4 ml glycerol, 2.31 g $KH_2PO_4$, 12.54 g $K_2HPO_4$ per 1 L)
- LBBM (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, 2.31 g $KH_2PO_4$, 12.54 g $K_2HPO_4$, 0.94 g $MgCl_2$ per 1 L)
- LBBNM (10 g bacto-tryptone, 5 g yeast extract, 30 g NaCl, 2.31 g $KH_2PO_4$, 12.54 g $K_2HPO_4$, 0.94 g $MgCl_2$ per 1 L)
- LBBMG (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, 2.31 g $KH_2PO_4$, 12.54 g $K_2HPO_4$, 0.94 g $MgCl_2$, 4 ml glycerol per 1 L)
- LBBSMG (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, 2.31 g $KH_2PO_4$, 12.54 g $K_2HPO_4$, 0.94 g $MgCl_2$, 4 ml glycerol, per 1 L). The different amounts of sorbitol was used during optimization: 36, 72, 110 and 144 g per liter of the medium
- MX (10 g bacto-tryptone, 2.31 g $KH_2PO_4$, 12.54 g $K_2HPO_4$, 0.94 g $MgCl_2$, 4 ml glycerol, 72 g sorbitol per 1 L). The different amounts of yeast extract was used during optimization: 5, 7.5, 10 and 15 g per liter of the medium

### 2.5. Determining optimal duration of the expression

For this experiment, MX medium was used. After induction, 2 ml of culture was sampled eight times during expression for the cell density measurements ($OD_{600}$). The value of pH of culture medium was adjusted to 7.5 by 1 M KOH, and 0.1 mM IPTG was added every 24 h. A total duration of the expression was 72 h.

### 2.6. Heat-shock pre-induction procedure

1 ml of the overnight culture was 50-times diluted in several 250 ml-glass flask with 50 ml in a fresh MX medium containing 25 μg/mL of kanamycin. The culture was cultivated at 37 °C until the cell density was reached an $OD_{600}$ of 0.6—0.8, and then incubated at 42 °C for 30 min without shaking. After that, the culture was cooled to room temperature and induced by the addition of 0.2 mM IPTG.

### 2.7. Cell extract preparation and analysis

Bacterial cells from culture was harvested by centrifugation at 4000*g* for 10 min. Bacterial biomass was resuspended in 20 mM sodium phosphate buffer (pH 7.0) with 100 mM NaCl and lysed by ultrasonic treatment on ice. The obtained extract was centrifuged at 10,000*g* for 20 min to sediment a cell debris.

Enzyme activity of the α-PsGal was determined with 2 mM of 4-NGP (4-nitrophenyl α-D-galactopyranoside) in 20 mM sodium phosphate buffer (pH 7.0) with 100 mM NaCl at 20 °C. One unit of the α-galactosidase activity was defined as amount of the enzyme that hydrolyzed 1 μmol of 4-NGP per min. Total quantity of *p*-nitrophenol was determined spectrophotometrically at 400 nm ($\varepsilon_{400} = 18{,}300\ M^{-1}\ cm^{-1}$).

Total protein was quantified according to Bradford method [13]. BSA was used as protein standard.

*E. coli* Rosetta (DE3)/40Gal transgenic strain productivity was determined as ratio of the total α-PsGal activity (units) in the cell extract to the cell density value ($OD_{600}$) measured before the cell harvesting (Rosetta (DE3)/40Gal productivity = α-PsGal units/$OD_{600}$).

### 2.8. Statistics

All values presented in this article are representative of at least three independent experiments. Data were analyzed using the Student's *t*-test of the SigmaPlot 2000 version 6.0 program (SPSS Inc.). Differences from controls were considered significant at $p \leq 0.05$.

## 3. Results and discussion

### 3.1. Construction for α-PsGal production

pET-40b (+) (Novagen) was selected for α-PsGal expression. It has N-terminal signal sequence that is specific for *E. coli* and allows directing a foreign protein to the cell periplasm. This plasmid has the gene encoding redox protein DsbC upstream recombinant protein gene that promotes the formation of disulfide bounds if necessary and correct folding of the recombinant protein

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