Betaine biosynthesis in a heterologous expression system based on the B12 producer *Pseudomonas denitrificans*

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Abstract. Glycine betaine is an important donor of methyl groups in various metabolic processes of the cell and acts as an osmoprotector when exposed to various abiotic stresses in pro- and eukaryotic organisms. Moreover, exogenous application of betaine activates the production of vitamin B12 in industrial strains-producers. In this work, we have developed a new technology for microbiological betaine synthesis that can be used in biotechnology to activate B12 biosynthesis during large-scale fermentation of *Pseudomonas denitrificans*.

1 Introduction

Vitamin B12 (cyanocobalamin): the vitamin is an antianemic factor. In the animal body, it plays a significant role in the process of hematopoiesis, the work of the red bone marrow and the biosynthesis of nucleic acids. Insufficient supply of vitamin B12 in pigs, poultry, and young ruminants causes malignant anemia (impoverished blood), accompanied by a sharp decrease in productivity, growth cessation, and complete exhaustion of the body due to low absorption of proteins and plant-based feed.

Known producers of cobalamin are prokaryotes and, above all, propionic and methane-forming bacteria, which also form this vitamin under natural conditions. High cobalamin titers (up to 200 mg/l) were achieved for the optional anaerobe *Propionibacterium shermanii*, but the most common producer in the industry today is *Pseudomonas denitrificans*, which produces similar titers of the target product - B12 [1].

Such well-known inducers of vitamin synthesis as betaine, choline, and cobalt can make a significant contribution to optimizing the cultivation conditions of *P. denitrificans* as a B12 producer [2]. Usually, to increase B12 production, chemically pure betaine is added to the culture medium of the producer at a concentration of 5-7 g/l during 50-140 hours of fermentation [3]. However, the need for constant application of exogenous betaine leads to

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an increase in the cost of production of B12 [4;5]. As one of the alternative sources of betaine, its intracellular synthesis by a recombinant B12-producing bacterium can be used, for example, due to the heterologous expression of its biosynthesis genes from available substrates.

Despite *de novo* inability to synthesize betaine, bacteria are able to enzymatically convert choline to betaine in a two-step enzymatic reaction. At the same time, choline itself, transported to the cell through systems with high and low affinity - BetT and ProU, respectively [6], is first oxidized to glycine-betaine-aldehyde by the enzyme choline dehydrogenase (BetA). The second stage of oxidation, catalyzed by glycine-beta-aldehyde dehydrogenase (BetB), then converts glycine-beta-aldehyde to glycine betaine [7]. However, the availability of choline - a substrate for the synthesis of glycine betaine is a limiting factor in the biosynthesis of this compound [8].

Recently, an alternative pathway for betaine biosynthesis has been described in which the available amino acid, glycine, is used as a precursor [9]. A new pathway was discovered in the halotolerant cyanobacteria *Aphanothece halophytica* and it consists of a 3-stage sequential reaction of glycine methylation, which is carried out by a pair of N-methyltransferase enzymes. One enzyme [*A. halophytica* glycine sarcosine methyltransferase (*ApGSMT*)] catalyzes the methylation reaction of glycine to sarcosine and sarcosine to dimethylglycine, respectively, and the other [*A. halophytica* dimethylglycine methyltransferase (*ApDMT*)] catalyzes the specific methylation of dimethylglycine to betaine [10].

2 Materials and methods

A DNA fragment containing the chimeric *GSMT/DMT* gene was obtained by polymerase chain reaction (PCR) using *A. halophytica* DNA as a matrix. *ApGSMT*-XbaI-dir and *ApGSMT*-linker-rev primers were used to obtain the *ApGSMT* gene, where *ApGSMT*-XbaI-dir is a primer specific to the N-terminal sequence of *ApGSMT*, including the site for XbaI restriction; *ApGSMT*-linker-rev is a reverse primer specific to the C-terminal sequence of *ApGSMT*, including the GGGGS linker sequence:

ApGSMT-XbaI-dir: 5'-TATCTAGAATGGCTATCAAAGAAAAAC-3'

ApGSMT-linker-rev: 5'-

CAGAGCCACCTCCGCCTGAACCGCCACCACCTGAATCTTTTTCGCAACGTG-3'.

To obtain the *ApDMT* gene, primers *ApDMT*-linker-dir and *ApDMT*-HindIII-rev were used, where *ApDMT*-linker-dir is a primer specific to the N-terminal sequence of *ApDMT*, including the GGGGS linker sequence; *ApDMT*-HindIII-rev is a reverse primer specific to the C-terminal sequence of *ApDMT*, including the site for HindIII restriction:

ApDMT-linker-dir: 5'-

CAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGACTAAAGCAGACGCAGTCGC-3'

ApDMT-HindIII-rev: 5'-TTAAGCTTCTAGGGTTTGTGGAAGTTG-3'.

After amplification, PCR products were mixed and repeated PCR was performed with *ApGSMT*-XbaI-dir and *ApDMT*-HindIII-rev primers.

The resulting fragment of the *GSMT/DMT* chimeric gene and pMF230 plasmid DNA were treated with XbaI and HindIII restriction enzymes, then cross-linked using a ligase reaction. The reaction mixture was used to transform competent *E. coli* Rosetta (DE3) cells. *E. coli* cells were used to isolate pMF230-GSMT/DMT DNA (Fig. 1).

Recombinant *P. denitrificans* strain-producer was obtained by electroporation of *P. denitrificans* CCUG 1783 cells with recombinant pMF230-GSMT/DMT plasmid. Colonies of *P. denitrificans* were collected from the surface of LB-agar and cultured for a day in a liquid LB-medium. To determine the productivity of the strain *P. denitrificans*/pMF230-

GSMT/DMT cells were incubated in a three-liter flask in a liquid LB-medium for a day at 25°C with intensive stirring (200 rpm). The biomass was separated from the culture medium by centrifugation and washed with saline on filter paper. The suspension was disintegrated in a mixture of methanol/water 1/1 (V/V). The resulting suspension was incubated at room temperature in an ultrasonic bath for 1 hour. After incubation, the suspension was centrifuged at 17000 g for 20 minutes. After that, the transparent supernatant was selected and transferred to new tubes for repeated centrifugation under the same conditions, followed by the selection of the supernatant for HPLC and MS analysis.

Identification of the obtained recombinant polypeptide is carried out according to characteristic mass spectrum peaks of glycine betaine: $[M+H]^+=118.1$ and $[2M+H]^+=235$ using the device Bruker HCT Ultra core facilities in the CUS of Biotechnology and Genetic Engineering FSC of Biodiversity FEB RAS.

Fig. 1. Recombinant plasmid DNA pMF230-GSMT/DMT, carrying a chimeric sequence consisting of the coding part of the *ApGSMT* genes and fused together using a linker sequence.

3 Results and discussion

At the first stage of our work, we created a chimeric gene (*GSMT/DMT*) consisting of fused *ApGSMT* and *ApDMT* coding sequences connected by a flexible linker. The chimeric gene was placed in a plasmid pMF230 with a wide range of hosts [11], the expression of which is controlled by a strong synthetic tac promoter [12]. Previously, it was shown that the joint heterologous expression of these two enzymes in the cyanobacterium *Synechococcus* sp. PCC 7942 and *Arabidopsis thaliana* and *Oryza sativa* plants leads to the accumulation of a significant amount of betaine, the level of which significantly exceeded the indicators of transgenic cells expressing enzymes of the choline-dependent pathway [13; fourteen].

To test the assumption that the chimeric *GSMT/DMT* gene can induce betaine biosynthesis, the recombinant plasmid pMF230-GSMT/DMT was transferred to *P. denitrificans* cells by electroporation. After the selection of transformed colonies, the recombinant strain was planted in a 1 l storage culture. Like most prokaryotes, members of the *Pseudomonas* genus are not able to synthesize choline and betaine de novo [15], and exogenous choline transporters are ubiquitous in this group based on analysis of current genome sequences [16].

The HPLC-MS method was used to identify betaine in a recombinant strain of *P. denitrificans*. To identify the peak, the glycine betaine standard was used (Fig. 2), as well as

by analyzing the decay products (daughter ions) (Fig. 3). Thus, we have shown that heterologous expression of the *GSMT/DMT* chimeric gene can provide a high level of betaine accumulation in the recombinant *P. denitrificans* strain. It is important to note that both in the original bacterium and in transgenic organisms carrying the GSMT and DMT enzymes, betaine synthesis is performed *de novo* and the addition of exogenous glycine did not significantly affect its production [17]. It has been shown that the accumulation of betaine in pro- and eukaryotic cells significantly increases their resistance to abiotic stresses [18].

Fig. 2. Chromatography of the isolated ion; Intens – relative value of the ion current intensity.

Fig. 3. MS and MS2 spectrum of ions with a molecular weight of 118 and 235.

4 Conclusions

Glycine betaine protects cells from negative abiotic stress factors by maintaining osmotic balance with the environment and stabilizing the quaternary structure of proteins. In addition, betaine is a supplier of methyl groups in general metabolism and activates the biosynthesis of an important anti-anemic vitamin - B12. However, the availability of a choline substrate for the synthesis of glycine betaine is a limiting factor in the production of this compound. Using heterologous expression of the GSMT/DMT chimeric gene in *P.*

denitrificans cells, a high betaine content was achieved by *de novo* biosynthesis. This work shows for the first time a method for bioengineering betaine synthesis based on the use of an artificial protein with several catalytic functions. It is important to note that recombinant cells use the available amino acid glycine as a substrate for betaine biosynthesis in the developed technology, rather than molecules of choline that is deficient for bacteria. The described approach can be used in the biotechnological industry to increase the resistance of recombinant bacterial strains to adverse abiotic factors, as well as to activate the synthesis of valuable metabolites, such as cobalamin.

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