Influence of nutrient medium and time on lipopeptide synthesis in *Bacillus subtilis* GM5

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Abstract. The level of expression of *srfAD* gene, a subunit AD of surfactin synthetase, and the yield of the total lipopeptide fraction were studied in relation to the composition of nutrient medium and the growth stage of the probiotic strain *Bacillus subtilis* GM5. The maximum concentration of the total lipopeptide fraction was observed during the growth of strain GM5 on SMN medium with soybean flour. The relative expression of the *srfAD* gene of *B. subtilis* GM5 was quantified by RT-qPCR. We conclude that the increase in the concentration of the total lipopeptide fraction on SMN medium correlates with the expression of the *srfAD* gene during 96-hour cultivation of GM5, which may indicate the production of mainly lipopeptide from the surfactin group ~~by~~ under these conditions.

1 Introduction

Bacillus subtilis bacteria are widely used in poultry farming as probiotics. They exhibit antagonistic activity against pathogenic microflora of the gastrointestinal tract (GIT) due to the production of secondary metabolites, they are also able to improve feed digestibility and enhance immunity of farm birds [1]. Various metabolites produced by probiotic bacteria of the genus Bacillus play an important role in the manifestation of these functions. According to the Natural Products Atlas 2.0, different species of *Bacillus* produce at least 455 secondary metabolites, among which lipopeptides, polyketides, bacteriocins, lantibiotics, siderophores and macrolactones are the dominant ones [2].

Bacterial lipopeptides have a number of advantages over synthetic compounds, including: low toxicity to humans, high biodegradability, digestibility, biocompatibility, low irritability, and a variety of chemical structures and properties. They find wide applications in various fields of biotechnology [3]. Based on the cyclic structure of the molecule, lipopeptides are subdivided into surfactin, iturin and phengicin families synthesized by non-ribosomal peptide synthetases (NRPS) or hybrid polyketide synthases/NRPS. Lipopeptide biosynthesis is regulated by several genes and is dependent on environmental conditions such as pH, temperature, and oxygen availability [4, 5].

Bacterial cyclic lipopeptide surfactin, a representative of the heptapeptide group, is an effective biosurfactant and exhibits antimicrobial and hemolytic activity. The synthesis of

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this lipopeptide is controlled by the *srfA* operon (*srfAA, srfAB, srfAC*, and *srfAD*) under the autoinducible Psrf promoter triggered by quorum sensing signaling molecules [6, 7]. Previously, the biological significance of the *srfA* gene in surfactin synthesis by *B. subtilis* bacteria was described by different scientific groups [8, 9]. Analysis of the *Bacillus tequilensis* ANSKLAB04 genome revealed that the *srfD* gene product is an important enzyme because it initiates surfactin formation. The *srfA* operon has also been shown to play an important role in post-translational modifications of surfactin synthetase [10]. It is known that *srfAD* represents the TE domain and is responsible for the regeneration of incorrectly bound T domains in the modules of the first three subunits [7, 11].

The aim of this work was to evaluate the level of *srfAD* gene expression and the yield of total lipopeptide fraction depending on the nutrient medium composition and growth stage of *B. subtilis* GM5.

Previously, *B. subtilis* strain GM5 (NCBI NZ_NKJH00000000) with fungistatic and antibacterial activity [12] and capable of producing lipopeptides [13] was isolated from the rhizosphere of potato (Solanum tuberosum). The following NRPS gene clusters were identified in the genome of *B. subtilis* strain GM5 using the antiSMASH program: BGC0001089_c1 Bacillaene; BGC0001184_c1 Bacilysin; BGC0000309_c1 Bacillibactin; BGC0000433_c1 Surfactin, BGC0001095_c1 Fengycin, BGC0000407_c1 Plipastatin [14]. The gene cluster of surfactin biosynthesis genes of *B. subtilis* strain GM5 in comparison with the closest surfactin homolog of *Bacillus velezensis* FZB42 is presented in Figure 1.

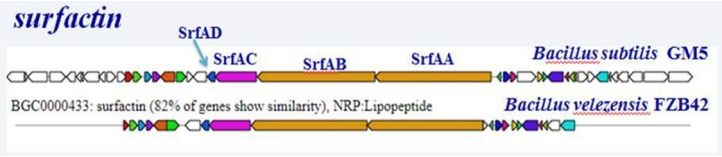

Fig. 1. Surfactin biosynthesis gene clusters of *B. subtilis* GM5 and *B. velezensis* FZB42 strains

2 Materials and Methods

B. subtilis strain GM5 was used as a lipopeptide producer.

The growth dynamics and accumulation of the total lipopeptide fraction of *B. subtilis* GM5 bacteria were studied on the following media: 1) Soybean Medium Nutrition (SMN) [15]; 2) LB (Luria broth) [15]. *B. subtilis* GM5 bacteria were cultured in a thermostat shaker (IKA®KS 4000, Germany) at 37 °C and a rocking intensity of 200 rpm for 96 h. The optical density (OD) of the bacteria was determined every 24 h on a spectrophotometer (Bio-Rad, USA) at a wavelength of 590 nm.

Synthesis, accumulation, and secretion of the total lipopeptide fraction were performed as we described previously [16]. Weight (gravimetric) analysis was used to calculate the concentration of lipopeptides [17].

Cells of strain GM5 grown in SMN and LB for 24, 48, 72, and 96 h were used for RNA secretion using ExtractRNA reagent (BC032, Eurogen, Russia). DNase I, RNase-free kit (Thermo Scientific, USA) was used to remove contaminating genomic DNA. RNA concentration was determined using a NanoPhotometer NP80 spectrophotometer (Implen, Germany). All RNA samples were diluted to a concentration of 50 ng/μL before use. Sequence analysis of genomic loci responsible for synthesis of B. subtilis GM5 NRPS was performed using the BLAST software package (http://www.ncbi.nlm.nih.gov). Primers were designed based on the annotated nucleotide sequences of srfAD and 16S rRNA genes

using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primer sequences are summarized in Table 1.

Table 1. Primer sequences of the genes used for the RT-PCR.

Gene	Primer sequence (5'-3')	Product
srfAD	F:TGGGCGGAATGATCACCTTC R:GGGCCAGATCGTAAAGCTCA	surfactin synthetase *srfAD*
16S rRNA	F:GTTGGTGAGGTAACGGCTCA R:TGCTCCGTCAGACTTTCGTC	16S ribosomal RNA

The expression of srfAD and 16S rRNA genes was analyzed by RT-qPCR using a commercial OneTub RT-PCR SYBR kit (Eurogen, Russia) and a real-time PCR system.

All reactions were performed in accordance with the manufacturer's protocol on a CFX96 Touch amplifier (Bio-Rad, USA). The annealing temperature of the target gene primers was 60 °C. The Cq of each gene was determined. The relative expression of srfAD gene was calculated using the $^{\Delta\Delta Ct}$ method, and the amount of target gene was calculated using the $2^{-\Delta\Delta Ct}$ formula [18]. Normalization was performed using the 16S rRNA reference gene. Samples of strain GM5 taken at 0 h were used as control to calculate gene expression.

Statistical processing of experimental data was performed by determining arithmetic averages and their standard deviations in Microsoft Excel 2016 program [19]. All experiments were performed in three repetitions.

3 Results

The dynamics of bacterial growth and accumulation of the total lipopeptide fraction of *B. subtilis* strain GM5 were studied on LB and SMN media.

The stationary growth phase of strain GM5 was observed after 24 h of growth on both media, with the optical density of the culture amounting to 1.88 units on LB medium and 1.77 units on SMN (Fig. 2). The pH of nutrient medium remained around 6.63 and 8.32 until 24 h when GM5 strain was cultured on SMN and LB media, respectively, then gradually increased, reaching 7.63 and 9.21 at 96 h of culturing (Fig. 2).

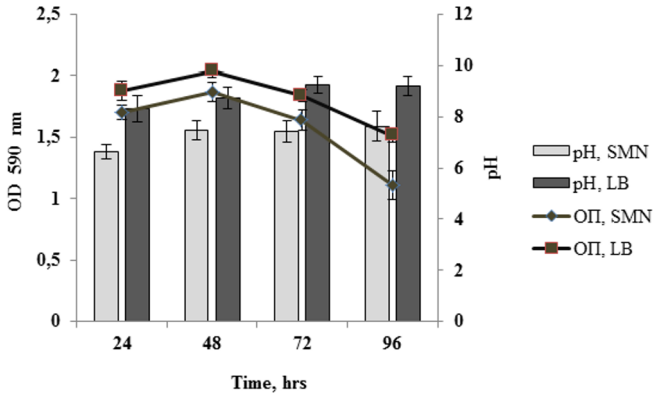

Fig. 2. Cultivation of *B. subtilis* GM5 cells on LB and SMN nutrient media at 37°C.

It was shown that the concentration of the total lipopeptide fraction gradually increased on both media, but on SMN medium it was significantly higher, reaching maximum values by 96 h of cultivation (Fig. 3). The concentration of lipopeptides on LB medium was 2-5 times lower compared to SMN medium.

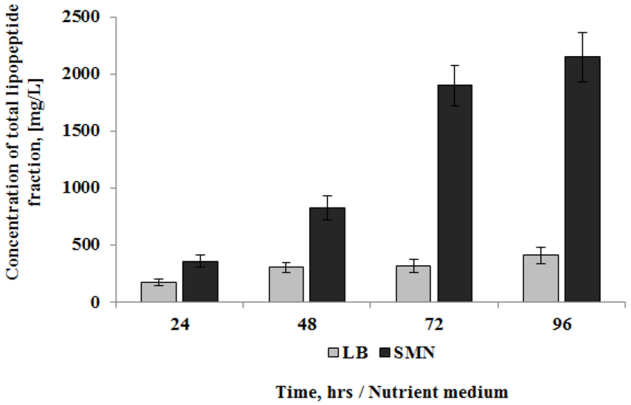

Fig. 3. Comparison of concentrations of the total lipopeptide fraction of *B. subtilis* strain GM5 in nutrient media LB and SMN at different hours of cultivation.

The relative expression of the *srfAD* gene, the AD subunit of surfactin synthetase of B. subtilis strain GM5 cultured on LB and SMN media, was quantified by RT-qPCR. When analyzing the expression changes of the *srfAD* gene, the control level was taken as 1 on the y-axis for a more accurate representation. Using the 16S rRNA gene as a reference, the gene under study was found to be expressed to different degrees when strain GM5 was cultured on different media (Fig. 4) and depending on the time of bacterial growth. In the case of bacterial growth on LB medium, no significant differences in the level of *srfAD* gene expression at different hours of cultivation were detected, while on SMN medium the gene expression was significantly higher than on LB medium and it increased at late hours of bacterial cultivation, which correlates with an increase in the concentration of lipopeptides in the medium (Fig. 4).

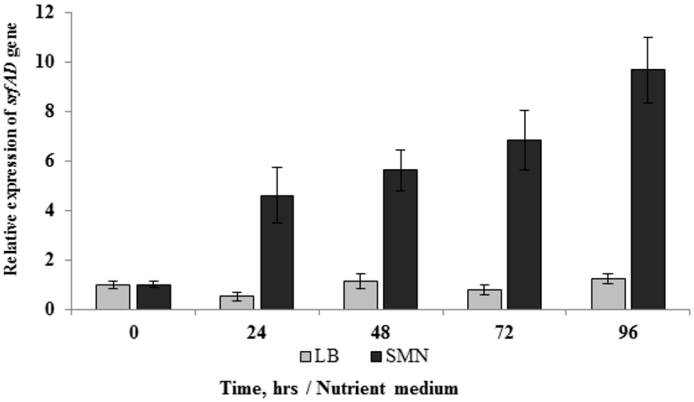

Fig. 4. Analysis of srfAD gene expression during growth of *B. subtilis* strain GM5 on LB and SMN nutrient media at 24, 48, 72, and 96 h. Relative gene expression was calculated using the $^{\Delta\Delta Ct}$ method and 16S rRNA as a reference gene.

4 Discussion

Many authors have shown that Lundy's nutrient medium, which contatin glucose and glutamic acid, is one of the most commonly used culture medium for surfactin synthesis. Research has been conducted to optimize this medium for various strains of *B. subtilis* [20]. However, there is a trend in biotechnology towards using low-cost mediums based on cheap substrates, such as waste or agro-industrial by-products, which makes possible to obtain large volumes of microbial agents or their bioactive metabolites at low cost, while maintaining the effectiveness of biological control. The components of low-cost media must provide sufficient energy for cellular biomass production and metabolite biosynthesis. This approach is increasingly being investigated as it makes possible to provide sustainable synthesis of surfactins [21].

The chemical composition of the nutrient medium and cultivation conditions affect cell growth and lipopeptide synthesis. Unlike other bacterial secondary metabolites, lipopeptide synthesis is induced when producers are depleted of one or more important nutrients [22, 23]. It was found that on different nutrient media with the same carbon content, the decrease in surfactin occurred at the same time, which may be due to the use of surfactin as a carbon source after glucose depletion [24]. In addition to carbon substrate concentration, the concentration of N, P, Na, Mg, Fe, Zn and Mn ions in the medium has also been shown to influence the level of lipopeptide synthesis [22]. In the present work, we have shown that SMN medium is optimal for the accumulation of total lipopeptide fraction (2147 mg/L) by *B. subtilis* strain GM5 bacteria. Soybean meal added to SMN medium serves as an inexpensive source of carbon, nitrogen and phosphorus, mannitol as carbon, and sodium salts as nitrogen. The SMN medium also contains Mn^{2+} salt, which it is well known to have a positive effect on increasing surfactin yields [25].

5 Conclusions

Thus, the increased concentration of the total lipopeptide fraction on SMN medium has been shown to correlate with the expression of the *srfAD* gene at 96 h of cultivation of strain GM5. This might indicate that *B. subtilis* produce mainly lipopeptide of the surfactin group under these conditions.

6 Acknowledgements

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