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Enhanced production of virginiamycin with the maintained optimal ratio of its components by a mutant *Streptomyces virginiae* IB 25–8 strain

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ABSTRACT

Virginiamycin, a widely used veterinary antibiotic produced by *Streptomyces virginiae*, represents a natural mix of two macrocyclic peptidolactones; its main components are the M1 and S1 factors, which act synergistically when present in the optimum ratio of 75:25. Because of a large number of genes involved into the virginiamycin biosynthesis, the development of high-yield strains able to synthesize M1 and S1 at a synergistic ratio with the total productivity exceeding 4 g/L still remains a relevant problem. Using a multi-step random UV mutagenesis with the simultaneous control of the M1:S1 ratio in a final product, a new highly productive and genetically stable *S. virginiae* strain IB 25–8 was obtained. After the medium improvement combined with the addition of a Diaion™ HP21 resin absorbing up to 98.5% of the total virginiamycin, the productivity of the strain has reached 5.21 ± 0.16 g/L, while the M1:S1 ratio remained to be synergistic (75:25). The effect of various medium components, their concentration, and resin types on the M1:S1 ratio has been shown. The obtained strain is promising for the industrial use due to its high productivity and the optimal M1:S1 ratio. A pre-fermentation addition of the selected DIAION™ HP21 resin to fermentation medium increases the total yield and simplifies the further isolation and purification of virginiamycin.

1. Introduction

In the modern world, maintenance of a high meat and milk production rate is provided by active use of veterinary antibiotics, which production volume makes almost 2/3 of the global antibiotic production and is estimated as about $4 billion (Gelband et al., 2015; van Boeckel et al., 2014). The most popular veterinary antibiotics belong to the tetracycline, cephalosporin, aminoglycoside, destomycin, and streptogramin groups. The last one is of special importance due to biological properties of streptogramins, which are nontoxic, non-mutagenic, biodegradable, and are characterized by the lack of accumulation in animal tissues and a narrow antimicrobial spectrum concerning *Staphylococcus* bacteria (Cocito, 1979).

Virginiamycin, a streptogramin antibiotic produced by *Streptomyces virginiae*, represents a natural mix of macrocyclic peptidolactones M and S. The main components of the antibiotic are the M1 and S1 factors. One of the unique features of a virginiamycin biosynthesis is that *S. virginiae* simultaneously produces both M1 and S1 in a ratio providing their maximum synergistic activity (Di Giambattista et al., 1989). The maximum synergism is observed when M1 an S1 present in the optimum ratio of 75:25; in this case, the total antimicrobial activity of virginiamycin increases in 3.5–4 times (Mast and Wohlleben, 2014). This rare feature provides a lack of any significant resistance of microorganisms to virginiamycin even after its long-term use.

Virginiamycin has a bacteriostatic and bactericidal activity towards the majority of gram-positive and some gram-negative bacteria including *Clostridium perfringens*, *Staphylococcus* spp., *Micrococcus* spp., *Campylobacter* spp., *Listeria* spp., clamydia (*C. trachomatis, C. pneumoniae*), and mycoplasma (*M. pneumoniae*) (Grozina et al., 2014). In addition to the antimicrobial activity, virginiamycin also acts as a growth stimulator, since it optimizes the absorption and metabolism of nutrients, improves the state of a small intestine epithelium, and inhibits the synthesis of harmful toxins and metabolites by gut organisms (Feighner and Dashkevicz, 1987; Hoyzman et al., 2011; Cervantes et al.,

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2002). Today virginiamycin is widely used in ethanol fuel production (Arshad et al., 2011) and as an antibiotic feed additive for cattle and poultry providing better survival of young animals and birds, lower sickness rate, increased body weight gain, and improved nutrition efficiency (Ives et al., 2002; Singh et al., 2008; Shojadoost et al., 2013).

Virginiamycin is registered and actively used in 32 countries including USA, Russia, Brazil, and China. In the first half of 2014, the import of virginiamycin-based preparations into Russia made 8.32% of the total import of antibacterial veterinary preparations (Vetanalytic, 2015) and 40.6% of the total import of macrolide antibiotics (Lavrenova, 2016). To 2020, a predicted increase in the consumption of this antibiotic in North America, Europe, and Asia will make 6% (Market Research Reports, 2016).

To provide a sufficient production of virginiamycin, efficient technologies of its industrial production, isolation, and purification are required along with overproducing strains. As a rule, productivity of wild strains is unsufficient for industrial microbiology; for example, productivity of the original ATCC 13161 strain, which was first described as a virginiamycin-producing microorganism, is only 30 U/mL or ~48 μg/mL (de Somer and van Dijck, 1955). In general, according to various publications, productivity of developed industrial *S. virginiae* strains under laboratory conditions still remains rather low varying from ~150–180 U/mL (~0.28 g/L) to 1200 U/mL (~ 1.9 g/L) (Biot, 1984; Prikrylova et al., 1987; Zvenigorodskii et al., 2001; Zhang et al., 2011). A final virginiamycin yield can be additionally increased by the medium improvement and optimization of fermentation conditions, but even in this case productivity of existing industrial strains is reported within the range of 3–4 g/L (Biot, 1984; Zhang et al., 2011; Han et al., 2013), except for one patent describing a method to increase the virginiamycin output to > 5 g/L (Yong, 2015). Note that none of the mentioned authors reported about the control of the M1:S1 ratio during optimization of fermentation conditions and in the final product, though it is an important characteristics of the virginiamycin efficiency.

The purpose of this work was the development of a new overproducing *S. virginiae* strain and the improvement of medium and fermentation conditions to achieve a final virginiamycin output exceeding 5 g/L. Additionally, the effect of a medium composition on the M1:S1 ratio was studied.

2. Materials and methods

2.1. Virginiamycin-producing microorganism and media composition

Streptomyces virginiae VKPM Ac-790 from the All-Russian Collection of Industrial Microorganisms of the State Research Institute of Genetics and Selection of Industrial Microorganisms (Moscow, Russia) was used as a parental strain. The productivity of the strain was 0.6 g/L.

VKPM Ac-790 and its mutants were grown, maintained, and stored on agar medium consisted of the following components (g/L): agar, 20.0, corn starch, 20.0; KH_2PO_4, 0.5, $MgSO_4 \cdot 7H_2O$, 0.5, KNO_3, 1.0, NaCl, 5.0, $FeSO_4$, 0.01 (pre-sterilization pH 6.8–7.0). For the long storage, cultures were freeze-dried and stored in sealed ampoules in a refrigerator.

Vegetation medium for seed cultures consisted of the following components (g/L): glucose, 1.0; meat extract, 3.0; soluble starch, 10.0, yeast autolysate, 5.0; casein hydrolyzate, 5.0; $CaCO_3$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.08 (pre-sterilization pH 7.0–7.2).

The basic fermentation medium (BFM) consisted of the following components (g/L): corn gluten, 5.0; yeast extract, 1.0; meat peptone, 2.5; malt extract, 10.0; $CaCO_3$, 5.0; glucose, 5.0 (pre-sterilization pH 7.0–7.2).

2.2. UV mutagenesis

After a 7-day growth at 28 °C, mycelium was washed off the agar surface with sterile water and passed through a sterile cotton filter or Schott funnel (pore size 100 μm) to remove large mycelium conglomerates. A spore concentration was calculated using a Goryaev-Thoma counting chamber and adjusted to $1.5–2 \cdot 10^6$ spores/mL. The final suspension was placed under a 12.5-W UV lamp (250–280 nm, Mineralight, USA) at a distance of 40 cm. The radiation intensity during treatment was 0.25 mW/cm^2. After UV treatment, 0.1 mL of the suspension was inoculated onto Petri plate containing the agar medium, spread on the whole agar surface, and incubated for 6–7 days at 28 °C.

To determine the optimum exposure time, the survival rate and mutagenesis efficiency were assessed within the exposure range of 5–40 min. A survival rate of colonies was determined as the percentage of colonies grown in the treated variants comparing to the untreated control. Mutagenesis efficiency was determined by the percentage of morphologically changed colonies grown after the UV treatment.

After UV treatment, 10–20 mutant colonies, which morphology significantly differed from initial one, were selected for the further work. Selected colonies were reinoculated on agar medium, grown for 7 days, then fermented (see 2.3), and preliminary analyzed for their productivity by a thin-layer chromatography; the best mutants were additionally examined for their productivity and M1:S1 ratio by HPLC. Mutant strains characterized by a high virginiamycin production and better M1:S1 ratio were selected and used for the further mutagenesis/selection cycles.

2.3. Fermentation conditions

Spore suspensions were prepared from 7-day mutant cultures grown on agar medium and adjusted to the concentration of $1 \cdot 10^6$ CFU/mL. The suspension (0.1 mL) was inoculated into 50-mL Erlenmeyer flasks containing 10 mL of vegetation medium. Seed cultures were grown for 48 h on Innova 44 incubation shakers (New Brunswick, Germany) at 28 °C and 250 rpm (5-cm orbit) and then were reinoculated into 50-mL flasks containing 10 mL of fermentation medium. Fermentation was carried out for 96 h under the same conditions.

2.4. Fermentation medium improvement and synthetic resin evaluation

To increase virginiamycin production by a selected high-yield mutant strain, fermentation medium was sequentially improved via evaluation of the effect of medium pH, various carbon or nitrogen sources, and the sources of macro- and microelements on the strain productivity. The fermentation was carried out as described above with the optimizing of one factor at a time.

Four types of synthetic resins were assessed for their capability to improve the production of virginiamycin: polistyrene/divinylbenzene-based Diaion® HP20 and Diaion® HP21 (Sorbent Technologies, USA) and also Amberlite® IR120 (strong-acid cation-exchange resin based on sulfonated polystyrene) and Amberlite® IRA900 (strong-alkaline anion-exchange resin based on benzyltrialkylammonium), both manufactured by Acros Organics (Belgium). Tested resins were added to fermentation medium (20 g/L) prior sterilization.

2.5. Analysis of virginiamycin content in fermentation broth

Virginiamycin was extracted from fermentation broth by mixing with the equal volume of ethyl acetate followed by a 2-h incubation under constant stirring at a room temperature. Then the ethyl acetate layer was removed, samples were centrifuged at 12,000 rpm for 3 min, and 200 μL of a supernatant was taken from each sample, dried, dissolved in 400 μL of a mobile phase, and analyzed by HPLC (see below). To determine the level of virginiamycin absorption by tested resins, resin-containing fermentation broth was filtered through a stainless steel mesh (hole size < 0.1 mm), and the collected resin was washed with distilled water. The further virginiamycin extraction and sample preparation was carried out separately for the resin and filtered fermentation broth as described above.

Productivity of mutant strains was preliminarily assessed by a thin-layer chromatography using Silica gel 60 TLC plates (Merck, Germany) as described by Zvenigorodskii (2001). The mix of chloroform and methanol (80:20, v/v) was used as the mobile phase. The absorbance was measured at 254 nm.

For high-yield mutants, virginiamycin content in samples was determined by HPLC according to Gossele et al. (1991) with some modifications. The analysis was performed using an Agilent 1200 chromatographic system (Agilent Technologies, USA) with a Zorbax C18-SB column (5 μm, 250 × 4.6 mm, Agilent Technologies, USA); the flow rate was 1.0 mL/min at 40 °C. The sample volume was 10 μL. The mobile phase was acetonitrile/distilled water/acetic acid (550/450/0.1). The absorbance was measured at 220 nm. Standard preparations of virginiamycin M1 + S1 (Santa Cruz Biotech, USA) and its separate M1 and S1 components (Sigma-Aldrich, USA) dissolved in the mobile phase were used as reference samples. Retention times for M1 and S1 were 6.93 and 11.92 min, respectively. The duration of the chromatographic analysis was 25 min.

Virginiamycin M1 and S1 concentrations in culture broth samples were calculated using the following formula:

$$C_{virg} = \frac{S_{virg} \times C_{st} \times A}{S_{st}},$$

where S_{virg} is the virginiamycin M1 or S1 peak area on the chromatogram of the tested sample, S_{st} is the virginiamycin M1 or S1 peak area on the chromatogram of the standard sample, C_{st} is the reference sample congentration, g/L, and *A* is the dilution factor of the tested sample.

2.6. Strain identification

The parental and resulted mutant strains were genetically identified by a 16S rRNA sequencing. DNA isolation was carried out as described by Boulygina et al. (2002). A fragment of 16S rRNA gene was amplified by PCR using 11F (50-GTTTGATCMTGGCTCAG-30) and 1492R (50-TACGGYTACCTTGTTACGACTT-30) universal primers (Lane, 1991). Nucleotide sequences of PCR products were determined using a Big Dye Terminator kit v.3.1 (Applied Biosystems, Inc., USA) and an ABI PRIZM 3730 DNA sequencer (Applied Biosystems, Inc., USA) according to Sanger et al. (1977) and manufacturer's recommendations. The reading was performed in both directions using the above primers. The obtained data were compared to the NCBI GenBank database using a BLAST software.

3. Results

3.1. UV mutagenesis

First, the survival rate and the frequency of morphological mutations of *S. virginiae* colonies were assessed to determine the optimum exposure time providing the maximum number of morphological mutations and, at the same time, a sufficient survival rate of colonies. According to the obtained results, the maximum frequency of morphological mutations (20.2–25.5%) was observed for a 15- and 20-min treatment (Fig. 1). At the same time, a survival rate for 15-min exposure was significantly higher (0.04% vs 0.002% for 20-min exposure), so it was chosen for the further work.

A direct selection of overproducing mutants via the assessment of productivity of each strain would be laborious and time-consuming process. In this study, we used visual selection of colonies morphologically differing from the parental strain. Such type of selection is based on pleiotropic effects of mutations related to a significant productivity increase and the corresponding metabolic abnormalities resulting in some morphological changes (Li, 1976). Selected strains were assessed for their virginiamycin productivity and M1:S1 ratio, and the best ones were used for the further mutagenesis/selection cycles.

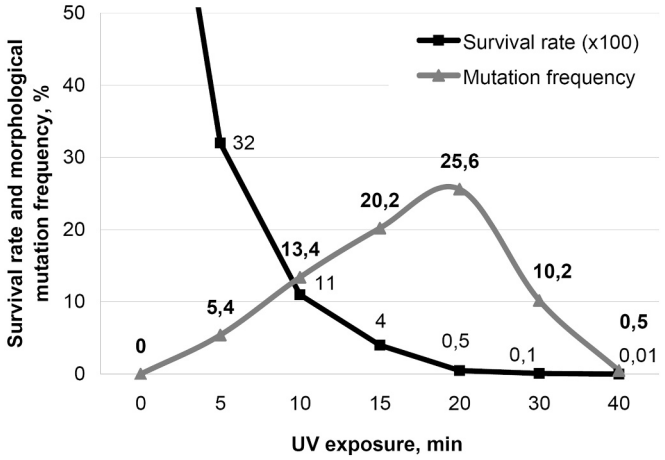

Fig. 1. Effect of the UV exposure time on the survival rate and morphological mutation frequency of *Streptomyces virginiae* VKPM Ac-790.

Finally, this multi-step mutagenesis resulted in the *S. virginiae* IB 25–8 strain, which virginiamycin productivity reached 2.35 ± 0.08 g/L that significantly exceeded that of the parental VKPM Ac-790. The M1:S1 ratio (75:25) in produced virginiamycin corresponded to the maximum synergism that became an additional advantage. The genetic stability of IB 25–8 was assessed by three successive subculturings with the productivity assessment during each subculturing. For this strain, the total virginiamycin yield and the M1:S1 ratio remained stable.

3.2. Morphological, physiological and biochemical properties of S. virginiae IB 25–8

S. virginiae IB 25–8 differed from VKPM Ac-790 by several morphological traits including the color of colonies grown on agar medium (grey with white border vs. uniform dark-grey, respectively), color of mycelium (dark-brown vs. beige), and the color of fermentation broth at the end of fermentation (dark-grey vs. brown).

3.2.1. Carbon source utilization

Unlike VKPM Ac-790, IB 25–8 was able to utilize not only glucose and maltose, but also sucrose (Table 1). In the case of other simple carbohydrates tested, no any growth of the strains was observed. Additionally, IB 25–8 was able to hydrolyze starch.

3.2.2. Nitrogen source utilization

No differences were observed between VKPM Ac-790 and IB 25–8 concerning their ability to utilize various nitrogen sources. IB 25–8 was unable to utilize urea and nitrates (including KNO_3), but utilized ammonium salts and organic nitrogen (see Section 3.4.3).

Table 1
Carbon source utilization ability of *Streptomyces virginiae* VKPM Ac-790 and IB 25–8 strains.

Carbon source	*Streptomyces virginiae* VKPM Ac-790	*Streptomyces virginiae* IB 25–8
Glucose	+	+
Arabinose	–	–
Xylose	–	–
Lactose	–	–
Galactose	–	–
Maltose	+	+
Sucrose	–	+
Fructose	–	–
Starch	–	+
Raffinose	–	–

Table 2
Antibiotic resistance of *Streptomyces virginiae* VKPM Ac-790 and IB 25–8 strains.

Antibiotic	Concentration, mg/mL	*Streptomyces virginiae* VKPM Ac-790	*Streptomyces virginiae* IB 25–8
Virginiamycin M1 + S1	0.6	+	+
	4.0	–	+
Tylosin 200	0.1	–	+
	0.5	–	+
Erythromycin	0.1	–	–
Lincomycin	0.1	–	–
Penicillin	0.1	–	–
Kanamycin	0.1	–	–
Tetracycline	0.1	–	–
Streptomycin	0.1	–	–
Gentamycin	0.1	–	–
Rifampicin	0.1	–	–
Chloramphenicol	0.1	–	–

3.2.3. Antibiotic resistance

Unlike the parental strain, IB 25–8 was resistant to increased concentrations of virginiamycin and tylosin 200 (4 and 0.5 mg/mL, respectively, Table 2).

3.3. Strain identification

To confirm the origin of IB 25–8, a 16 S rRNA sequencing of both parental and mutant strains was performed. Almost whole sequence (1463 nucleotides) of the bacterial component of the amplified fragment of gene encoding 16 S rRNA was determined for both strains. No any archaeal and minor DNA components were revealed. Nucleotide sequences of PCR fragments of both strains were identical between themselves. The obtained sequence for IB 25–8 was compared with similar sequences from the GenBank database. The maximum similarity level (99.4%) was revealed concerning the typical strain of *S. virginiae* NBRC 12827 (AB184175). According to the current standards (Stackebrandt and Ebers, 2006), such similarity level is sufficient to consider IB 25–8 belonging to the genera *Streptomyces*.

After the completion of the IB 25–8 identification and study of its biochemical and physiological properties, the strain was deposited to the All-Russian Collection of Microorganisms (Skryabin Institute of Biochemistry and Physiology of Microorganisms, Pushchino, Russia) under the accession no. VKM Ac-2738D.

3.4. Fermentation medium improvement

3.4.1. Effect of pH stabilization

A significant pH increase was observed after 50 h of IB 25–8 fermentation reaching 8.5 to the end of the process; at the same time, biosynthesis of virginiamycin was terminated at the pH level of 7.5–7.6 (Fig. 2). To avoid the revealed inhibiting effect, the effect of various pH stabilizers on the virginiamycin yield was studied. Inorganic salts (Table 3) were added to fermentation medium before sterilization. Fermentation time was 92 h. According to the obtained results, the maximum strain productivity (2.95 ± 0.09 g/L) was observed at pH 6.8 stabilized by the addition of Na_2HPO_4 and KH_2PO_4 (1.0 and 1.6 g/L, respectively; Table 3).

3.4.2. Effect of various carbon sources

Metabolic changes in mutant strains causing a productivity increase are also able to influence on the possibility and efficiency of utilization of various nutrients (Li, 1976). For each overproducing strain, an appropriate improvement of the quantitative and qualitative composition of fermentation medium should be carried out to realize its potential productivity. Therefore, a series of experiments was planned to determine the optimum sources of carbon, nitrogen, macro- and microelements, and their optimum concentrations providing better IB 25–8 productivity.

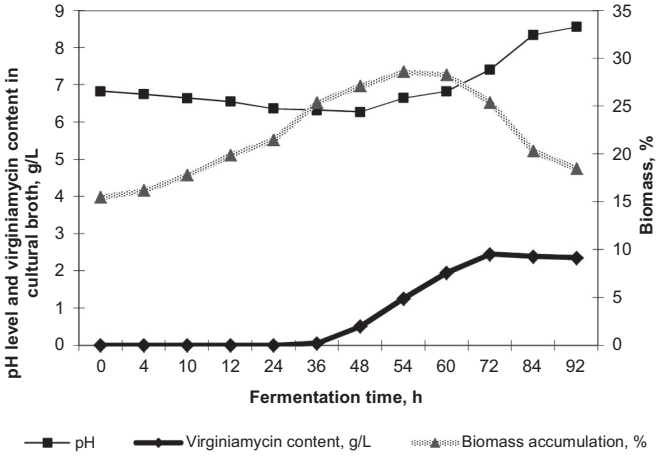

Fig. 2. Dynamics of pH changes, biomass accumulation, and virginiamycin content in fermentation broth during fermentation of *Streptomyces virginiae* IB 25–8.

Table 3
Effect of inorganic supplements on the pH stabilization and virginiamycin production by *Streptomyces virginiae* IB 25–8.

Supplement	Concentration, g/L	pH_{end} *	Virginiamycin content in fermentation broth, g/L
Control medium	–	8.56	2.35 ± 0.08
K_2HPO_4	0.5	7.11	2.37 ± 0.08
	1.0	6.98	2.45 ± 0.09
	2.0	6.77	2.34 ± 0.08
KH_2PO_4	0.5	7.85	2.25 ± 0.07
	1.0	7.35	2.35 ± 0.08
	2.0	7.39	2.34 ± 0.09
KH_2PO_4 + K_2HPO_4	1.6 + 1	6.68	2.55 ± 0.09
Na_2HPO_4 + KH_2PO_4	1 + 1.6	6.83	**2.95 ± 0.09**

* pH level at the end of fermentation.

At the first stage, the effect of four earlier determined carbon sources for IB 25–8 on the virginiamycin production and M1:S1 ratio was assessed. A control fermentation medium (CFM) represented BFM supplemented with KH_2PO_4 and Na_2HPO_4 (1.6 and 1.0 g/L, respectively). The results of the experiment are shown in Fig. 3. The maximum virginiamycin productivity (3.27 ± 0.10 g/L) was obtained for 35 g/L of sucrose; M1:S1 ratio was 72:28. A high productivity level was also observed for 20 g/L of glucose or starch (3.15 ± 0.07 and 2.95 ± 0.11 g/L, respectively); however, in both cases, the M1:S1 ratio was not synergictic (55:45 and 59:41, respectively).

3.4.3. Effect of various nitrogen sources

The majority of microorganisms are able to utilize nitrogen from inorganic compounds. At the same time, synthesis of biologically active substances in amounts exceeding the own needs of microorganisms requires an additional presence of organic nitrogen-containing compounds. Various flours or meals represent good sources of organic nitrogen. Along with proteins and amino acids, they contain carbohydrates, lipids, nucleic acids, microelements, and other compounds important for the synthesis of secondary metabolites. The effect of additional organic nitrogen sources on the productivity of IB 25–8 is shown in Fig. 4. A CFM corresponded to that of the Section 3.4.2 excepting the replacement of glucose (5.0 g/L) with sucrose (35.0 g/L). The maximum virginiamycin productivity (3.65 ± 0.11 g/L) was observed for the medium supplemented with 10 g/L of pea flour; in this

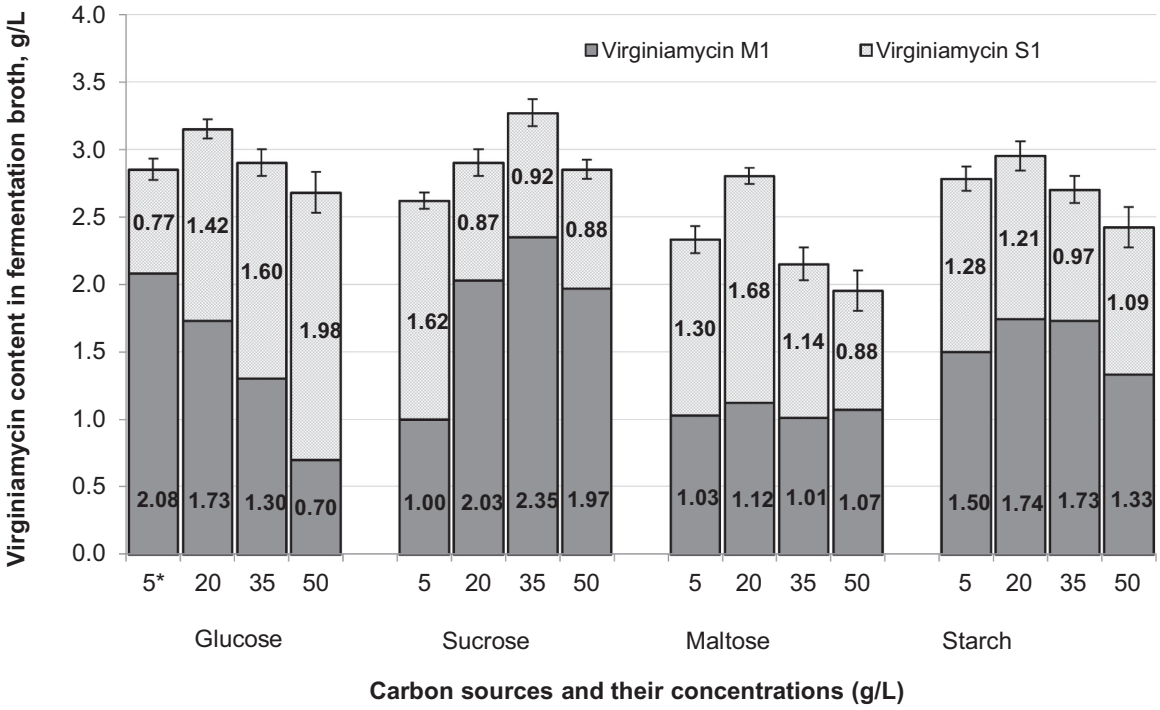

Fig. 3. Effect of various carbon sources and their concentrations on the biosynthesis of virginiamycin M1 and S1 by *Streptomyces virginiae* IB 25–8. Fermentation medium used as the control is indicated with asterisk. Here and in Figs. 3 and 4, standard deviations are shown for the total virginiamycin content (M1 +S1).

case, the M1:S1 ratio was 75:25 that corresponded to the maximum synergism. Significant deviations from the synergistic M1:S1 ratio were observed in the case of soybean flour (34–45: 55–66) and corn meal (48–52: 48–52).

Inorganic nitrogen sources represent important components of fermentation media for many microorganisms. Due to metabolic changes, mutant strains are often unable to utilize certain forms of inorganic nitrogen; therefore, the assessment of various sources of inorganic nitrogen is an important medium improvement stage. Results of such assessment are shown in Table 4. For all tested variants, M1:S1 ratio remained within the optimal limits (65–76: 25–35). The maximum total virginiamycin yield (3.91 ± 0.12 g/L) was observed for the medium supplemented with 1.0 g/L of $(NH_4)_2SO_4$.

3.4.4. *Effect of macro- and microelements addition*

Macroelements (P, S, K, Ca, Mg, etc.) form the main structural elements of cells and work as a part of their enzymatic systems. Therefore, they are involved into the most important physiological functions, such as regulation of the cell membrane permeability, energy transfer, enzyme activation, etc. Therefore, we studied the effect of

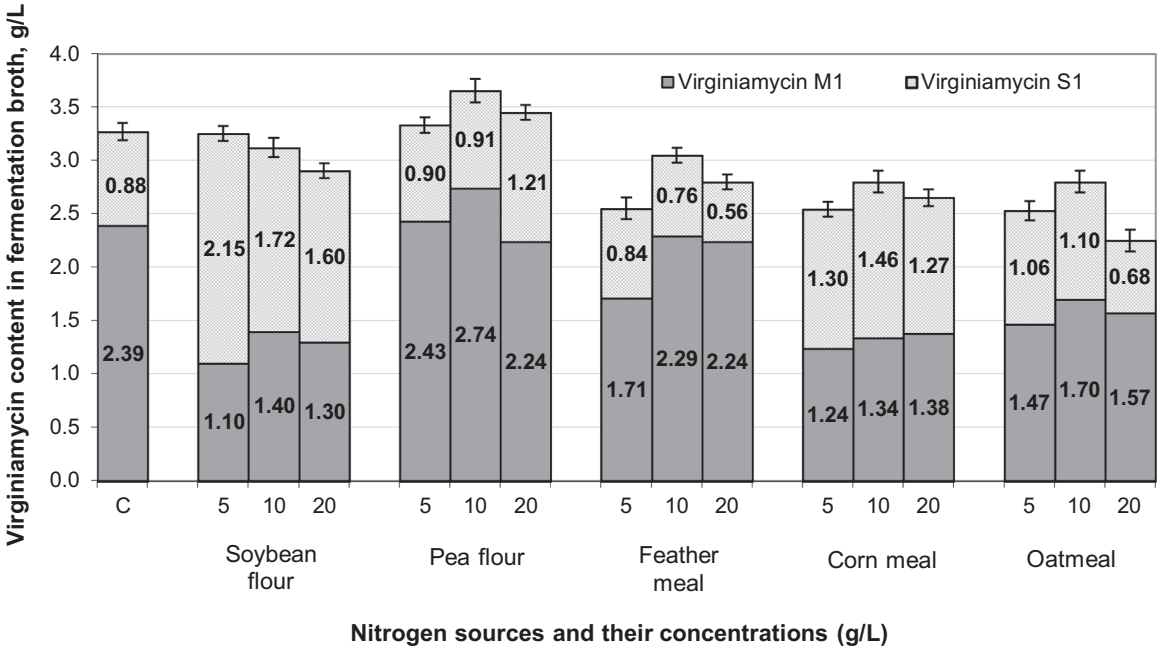

Fig. 4. Effect of various organic nitrogen sources and their concentrations on the biosynthesis of virginiamycin M1 and S1 by *Streptomyces virginiae* IB 25–8. *C*, control medium.

Table 4
Effect of medium supplementation with different sources of inorganic nitrogen on the virginiamycin productivity of *Streptomyces virginiae* IB 25–8.

Inorganic nitrogen source	Concentration, g/L	Virginiamycin content in fermentation broth, g/L
Control	–	3.58 ± 0.11
Ammonium sulfate $(NH_4)_2SO_4$	0.1	3.58 ± 0.11
	0.5	3.65 ± 0.11
	1	**3.91 ± 0.12**
	1.5	3.41 ± 0.10
Ammonium chloride NH_4Cl	0.1	3.22 ± 0.10
	0.5	3.48 ± 0.11
	1	3.66 ± 0.10
	1.5	3.26 ± 0.10
Ammonium citrate $C_6H_8O_7$*2NH_3	0.1	3.18 ± 0.10
	0.5	3.46 ± 0.10
	1	3.32 ± 0.11
	1.5	3.26 ± 0.10
Ammonium nitrate $NH_4(NO_3)_2$	0.1	2.85 ± 0.09
	0.5	2.87 ± 0.09
	1	2.64 ± 0.08
	1.5	2.46 ± 0.08
Urea (CH_4N_2O)	0.1	2.95 ± 0.10
	0.5	2.71 ± 0.09
	1	2.33 ± 0.07
	1.5	2.26 ± 0.07

For this experiment, control fermentation medium (CFM) corresponded to that of the Section 3.4.2 supplemented with 10 g/L of pea flour. Inorganic nitrogen sources were added to CFM prior sterilization.

Table 5
Effect of medium supplementation with different sources of macroelements on the virginiamycin productivity of *Streptomyces virginiae* IB 25–8.

Source of macroelements	Concentration, g/L	Virginiamycin content in fermentation broth, g/L
Control*	–	3.91 ± 0.12
$MgSO_4$	0.5	3.85 ± 0.11
	1.0	**4.06 ± 0.12**
	2.0	3.75 ± 0.12
NaCl	3.0	3.62 ± 0.12
	0.5	3.80 ± 0.12
	1.0	3.83 ± 0.12
	2.0	**4.08 ± 0.12**
KNO_3	3.0	3.77 ± 0.12
	0.5	3.40 ± 0.10
	1.0	3.55 ± 0.11
	2.0	3.70 ± 0.12
$NaNO_3$	3.0	3.69 ± 0.12
	0.1	3.35 ± 0.09
	0.5	3.45 ± 0.08
	1.0	3.25 ± 0.07
	3.0	3.21 ± 0.07

* For this experiment, control fermentation medium (CFM) corresponded to that of the previous section with addition of 1.0 g/L of $(NH_4)_2SO_4$. Macroelement sources were added into CFM prior sterilization.

addition of some macroelements (Na, Mg, K, S, and Cl) on a virginiamycin biosynthesis by IB 25–8. The obtained results are shown in Table 5. For all variants, M1:S1 ratio remained within the optimal limits (65–75: 25–35). The maximum virginiamycin yield was observed for medium supplemented with 1.0 g/L of $MgSO_4$ or 2.0 g/L of NaCl. A simultaneous addition of these two compounds in these concentrations provided virginiamycin yield equal to 4.17 ± 0.12 g/L.

Microelements (cobalt, manganese, copper, zinc, etc.) involved into the synthesis and activation of some enzymes are also required for the normal development of microorganisms. Microelements are contained in complex components of fermentation medium (pea flour, meat peptone, malt extract, etc.), but high-yield strains with changed metabolism often require an increased amount of these nutrients. The study of the effect of medium supplementation with additional source of microelements (stock solution including (g/L): $FeSO_4$, 0.24; $ZnSO_4$, 0.84; $MnCl_2{\cdot}4H_2O$, 0.42; $CuSO_4{\cdot}2H_2O$, 0.02) in a volume of 83 μL/L demonstrated only a small positive effect (strain productivity 4.30 ± 0.13 g/L).

3.5. Virginiamycin production enhancement by addition of synthetic adsorbing resins

Overproducing mutant strains are often undergone to the self-inhibiting effect of their metabolites produced in an increased volume and able to inhibit not only their own biosynthesis, but also the growth and development of a microorganism. One of the ways for effective reduction of possible cytotoxicity and self-inhibition effects is the fermentation in the presence of adsorbing resins (Phillips et al., 2013). However, this method is complicated by the necessity to select a certain type of resin for each metabolite, since even resins with similar physical characteristics can provide opposite results, either increasing, or reducing the content of a target product in fermentation broth (Warr et al., 1996; Lam et al., 1995). At the same time, a correct selection of an adsorbent can provide a very significant yield increase (Singh et al., 2010).

In this study we assessed four adsorptive polymeric resins of different nature, Diaion® HP20, Diaion® HP21, Amberlite® IR120, and Amberlite® IRA900. Fermentation medium composition was the following (g/L): sucrose, 35.0; pea flour, 10.0; corn gluten, 5.0; meat peptone, 2.5; yeast extract, 1.0; malt extract, 10.0; $CaCO_3$, 5.0; KH_2PO_4, 1.6; Na_2HPO_4, 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 1.0; NaCl, 2.0, and the stock microelement solution, 83 μL/L (pre-sterilization pH 7.0–7.2). Results of this experiment are shown in Fig. 5. The use of IRA900 and IR120 caused suppression of the virginiamycin biosynthesis by 47.0% and 30.5%, respectively; moreover, in the first case, the M1:S1 ratio was beyond of the optimum range. In addition, both resins almost did not adsorb virginiamycin from fermentation broth (12% and 3% of the total virginiamycin yield, respectively). At the same time, addition of HP20 and HP21 resins increased productivity of IB 25–8 by 9.4% and 22.6%, respectively, with the maintenance of the optimum M1:S1 ratio. The level of virginiamycin adsorption by HP20 and HP21 was 65% and 98.5%, respectively. The maximum virginiamycin yield was observed for the HP21 resin (5.21 ± 0.16 g/L); the M1:S1 ratio was 75:25.

Thus, after the completion of the whole series of experiments, the following composition of fermentation medium has been determined to provide the best virginiamycin productivity of IB 25–8 (g/L): Diaion® HP21, 20; sucrose, 35.0; pea flour, 10.0; corn gluten, 5.0; meat peptone, 2.5; yeast extract, 1.0; malt extract, 10.0; $CaCO_3$, 5.0; KH_2PO_4, 1.6; Na_2HPO_4, 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 1.0; NaCl, 2.0 g/L, and stock microelement solution, 83 μL/L (pre-sterilization pH 7.0–7.2).

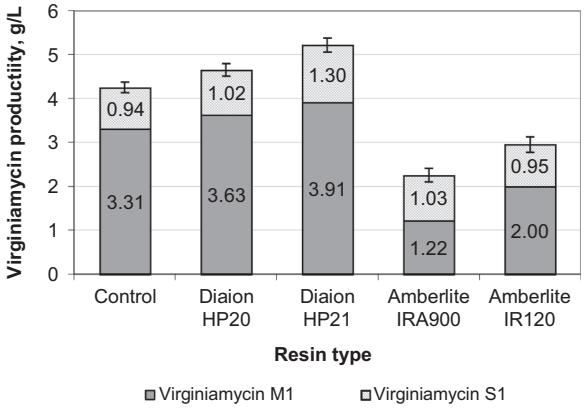

Fig. 5. Effect of a synthetic adsorbing resin addition on the virginiamycin M1 and S1 production by *Streptomyces virginiae* IB 25–8.

4. Discussion

Microbial production of biologically active substances requires a development of stable overproducing strains. Methods of classic selection, screening of wild strains, and directed mutagenesis are laborious and time-consuming. Modern approaches, which include either genetic engineering, or induced mutagenesis, are more productive. However, in the case of virginiamycin consisting of several components, which biosynthesis is regulated by a large group of genes (Pulsawat et al., 2009) and which antibiotic efficiency depends on the correct M1:S1 ratio, development of stable high-yield recombinant strains seems to be an extremely difficult task. An indirect evidence of this fact is the lack of any information about the development of genetically modified *S. virginiae* strains with improved production of virginiamycin M1 + S1; the existing publications on the genetic modification of this microorganism are connected mainly with the knockout or enhancement of the biosynthesis of either M1, or S1 (Takuya and Shigeru, 2005; Pulsawat et al., 2009).

For the same reasons, development of mutant overproducing strains, synthesizing both M1 and S1 components in the correct ratio, also seems to be a difficult task. For example, in the course of a long-term program of extensive selection of *S. virginiae* performed by the Phibro Animal Health Corp. (the main global manufacturer of virginiamycin-based formulations), the use of UV and chemical mutagenesis resulted in a wide range of mutant strains with different characteristics, including various deviations in the M1:S1 ratio or even completely blocked biosynthesis of one or both components (Lanoot et al., 2005). The aforesaid probably explains a very low number of publications and patents describing the development of overproducing *S. virginiae* strains; moreover, the most part of authors reports about the total yield increase, but do not informs about the control of the M1:S1 ratio. At the same time, use of high-yield strains producing virginiamycin M1 and S1 in the optimal 75:25 ratio would make it possible to exclude technological stages required to correct this ratio in a final formulation or to separate M1 and S1 components for their further mixing in the required proportion. As a result, industrial production of virginiamycin would be significantly simplified.

An additional and, sometimes, a significant increase in the strain productivity can be achieved by the improvement of a fermentation medium composition. There is a number of publications describing optimization of fermentation media by the selection of carbon (Zvenigorodskii et al., 2001) or nitrogen (Nomura et al., 1990; Zhang et al., 2011; Yong et al., 2015) sources or the medium supplementation with amino acids and nucleotides (Zvenigorodskii et al., 2001), virginiamycin precursors (Han et al., 2013), and biosynthesis regulators (Yang et al., 1995), providing a significant increase in the virginiamycin yield. However, almost all authors do not report about the M1:S1 ratio in the final product. This parameter was controlled only during the study of the effect of a virginiae butanolide-C (VB-C) addition to fermentation medium (Pulsawat et al., 2009); the reported M1:S1 ratio was far from the ideal in both presence and absence of VB-C (91:9 and 86:14, respectively). At the same time, this study, as well as our previous study with another overproducing *S. virginiae* strain (Savushkin et al., 2016), first showed that the M1:S1 ratio depends on the type and concentration of carbon and nitrogen sources; in some cases, relatively high virginiamycin yield was accompanied by a shift of this ratio far beyond the limits of its optimal range. Therefore, an additional control of this parameter during the development of overproducing *S. virginiae* strains and optimization of fermentation medium seems to be expedient.

In recent years, various adsorbing resins are widely used in the microbiological production of biologically active substances. However, as far as we know, there was no information about the use of such resins in the virginiamycin production excepting one patent (Yong, 2015), which described the application of an unidentified macroporous Dow resin combined with non-ionic surfactant to improve the permeability of cell membranes and gas exchange in fermentation medium in a 1000-L bioreactor. According to this patent, optimization of fermentation medium components combined with the medium supplementation with the resin and surfactant increased productivity of the used *S. virginiae* strain up to > 4000 U/L (> 6 g/L). Note that this patent does not contain information about the M1:S1 ratio in the final product and the initial productivity of the strain used. Moreover, authors do not propose the use of a resin as a way to bind virginiamycin from fermentation broth. Our study demonstrated the variability of the M1:S1 ratio depending on the resin type and allowed us to select the optimal virginiamycin sorbent providing a significant increase in the antibiotic output, while the M1:S1 ratio remained at the optimum level. An additional advantage of the selected Diaion® HP21 resin is that it adsorbs almost all (98%) produced virginiamycin that significantly facilitates its further separation and purification.

5. Conclusion

The performed study resulted in the development of a highly-producing *S. virginiae* IB 25–8 (VKM Ac-2738D) strain, optimization of a composition of fermentation medium, and selection of an adsorbing resin to provide a final virginiamycin output of 5.21 ± 0.16 g/L with the optimum M1:S1 ratio (75:25). Variations of the M1:S1 ratio in the final product depending on the type and concentration of major components of fermentation medium have been demonstrated. A high level of virginiamycin adsorption by the selected resin (98%) is able to facilitate the process of industrial production of this compound. The obtained results will be used for the further selection studies and the scaling-up of the virginiamycin production by IB 25–8 and may be of some interest for other researchers working in the field of the virginiamycin production.

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Author contribution

Study concept and design: Glagoleva and Dzhavakhiya. Strain development: Savelyeva, Popova, Grebeneva. Medium improvement: Glagolev, Savushkin. Strain productivity analysis: Novak. Manuscript drafting: Dzhavakhiya. Critical revision: Glagoleva, Ovchinnikov.

Financial disclosure

The authors declare there is no financial interest to disclose.

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