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ORIGINAL ARTICLE

Effect of adsorbing resins on the rapamycin biosynthesis by *Streptomyces hygroscopicus* VKM Ac-2737D

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Rapamycin, a metabolite of *Streptomyces hygroscopicus*, has a wide range of therapeutic applications in medicine. However, the possibility of its use is significantly limited, since the existing natural strains of this microorganism have rather low productivity that negatively influences the cost of the substance and the possible volumes of its industrial production. At the same time, the commercial use of overproducing strains is also very problematic, since they secrete a large number of metabolites suppressing both the growth of microbial culture and the biosynthesis of a target product. A possible way to solve the problem includes the addition of adsorbents to a fermentation medium for the binding of excess metabolites. In this study, five different selective adsorbing resins, DIAION™ HP20, DIAION™ HP21, DIAION™ HP 20SS, Chromolite, and LPS-500 were tested to provide optimal conditions for the rapamycin biosynthesis by the earlier developed highly-active *S. hygroscopicus* strain VKM Ac-2737D. The experiments were performed in a 15-L bioreactor using the optimized fermentation medium developed earlier and supplemented with the tested resins. The highest rapamycin yield (1420±5 mg/L) was obtained using a Chromolite 15AD2 adsorbing resin; this result exceeds the earlier obtained yield by 11.4%. The sorptive capacity of this resin was 92% that may significantly facilitate the processes of rapamycin isolation and purification under industrial conditions. The results of the study may be of certain interest for the further scaling-up of the industrial rapamycin production.

Keywords: rapamycin, *Streptomyces hygroscopicus,* antibiotics, secondary metabolites, fermentation, bioreactor, adsorbing resins

Introduction

Rapamycin, which is also known as sirolimus, is a secondary metabolite of the actinomycete bacterium *Streptomyces hygroscopicus* (Vezina et al., 1975). The structure of this nitrogen-containing compound belonging to macrolide antibiotics (Dutta et al., 2014) is shown in Fig. 1. Rapamycin is characterized by multiple effects on the organism, the main of which are the capability for cell division suppression used in the therapy of oncogenic diseases (Murugan, 2019) and immunosuppressive properties used to prevent graft rejection or any other immune diseases (Waldner et al., 2016; Yoo et al., 2017). Besides, some researchers consider rapamycin as a remedy for delaying aging and also for prevention and therapy of age-associated diseases, such as atherosclerosis, osteoarthritis, neurodegenerative ad heart diseases (Blagosklonny, 2017; Bagherpour et al., 2018).

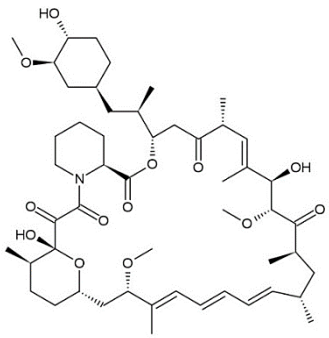

Fig. 1. Rapamycin structure.

Everolimus, a chemical derivative of rapamycin, is also widely used in the therapy of oncologic diseases. Everolimus actively inhibits the growth and proliferation of fibroblasts, as well as the tumor, endothelial, and unstriated muscle tissues of blood vessels and is considered a promising drug for the therapy of a progressive hormone-dependent HER2-negative breast cancer in women in a post-menopause period and the therapy of adult patients suffering from the renal angiomyolipoma, nonfunctional neuroendocrinal tumors of the gastrointestinal or pulmonary origin, and progressive neuroendocrinal tumors of the pancreas (Chan et al., 2010; Hasskarl, 2018).

In the first studies of the rapamycin biosynthesis by *S. hygroscopicus*, the productivity of strains cultured in a 250-L bioreactor for 96 h did not exceed 87 mg/L (Sehgal et al., 1975); in the case of 6-day cultivation in 500-mL flasks, it did not exceed 100 mg/L (Vezina, 1975). The analysis of existing publications and patents shows no significant changes in the situation: the productivity of existing industrial strains remains within 700–1000 mg/L (Zhao & Zhao, 2012; Cao et al., 2019) that increases the cost price of the substance and reduces possible volumes of its industrial production. Thus, the use of rapamycin in medicine is still limited, even despite its wide therapeutic potential. In this connection, the development of a strain with high biosynthetic activity concerning rapamycin and the development of technology suitable for the industrial production of this antibiotic represent very relevant tasks.

Earlier a highly active strain *S. hygroscopicus* R 33-41 was obtained at the Laboratory of Biotechnology of Physiologically Active Substances of the Federal Research Center "Fundamentals of Biotechnology" of the Russian Academy of Sciences (Moscow) by an undirected multi-step induced UV mutagenesis. The productivity of this strain reached 655±5 mg/L of rapamycin, more than 15-fold exceeding that of the initial strain *S. hygroscopicus* ATCC 29253 (Savelyeva et al., 2017a). The obtained strain was deposited in the All-Russian Collection of Microorganisms at the Skryabin Institute of Biochemistry and Physiology of Microorganisms with the accession number VKM Ac-2737D. The further optimization of the nutrient medium composition combined with the full experimental factorial planning, steepest ascent method, and the movement along the concentration gradient method provided an additional increase in the productivity of this strain under submerged cultivation in flasks or a bioreactor up to 1215 ± 5 (Savelyeva et al., 2017b) or 1275 ± 5 (Dzhavakhiya et al., 2019) mg/L of rapamycin, respectively, providing a 30-fold increase compared to the productivity of the initial strain *S. hygroscopicus* ATCC 29253.

Strains obtained by the non-directed induced mutagenesis and characterized by improved productivity to the target biologically active compounds are known to have impaired metabolic pathways (Egorov, 1989). Due to this fact, the cultivation of such strains is accompanied by the secretion of a large number of metabolites suppressing both the culture growth and the biosynthesis of a target compound (Phillips, 2013). One of the ways to reduce the cytotoxic activity of the secreted metabolites is the use of adsorbing resins during fermentation (Frykman et al., 2006; Phillips et al., 2013).

Adsorbing resins are widely used in the biotechnological production of biologically active compounds (Vojshvillo et al., 2007; Savushkin, 2016; Pathapat et al., 2018). Nevertheless, this method is not universal and requires some studies to choose the appropriate selective absorbent for a target metabolite, since the same adsorbent may provide different effects for different processes (Phillips et al., 2013). The correctly chosen adsorbent may significantly increase the yield of a target product (Jia et al., 2006; Zhang et al., 2012). For example, our earlier studies on the effect of adsorbing resins on the virginiamycin biosynthesis in *S. virginiae* VKM Ac-2738D showed the use of a Diaion HP21 resin increases the productivity of this strain (Dzhavakhiya et al., 2016). Taking into account a high need in the efficient rapamycin production technology suitable for a large-scale realization, the purpose of this study was the selection of a selective adsorbing resin providing optimal conditions for the rapamycin biosynthesis by *S. hygroscopicus* BKM Ac-2737D.

Materials and methods

Strain cultivation and storage

In this study the *Streptomyces hygroscopicus* strain VKM Ac-2737D obtained from the initial *S. hygroscopicus* strain ATCC 29253 by a multi-step selection combined with the use of mutagenic factors and the directed selection and deposited in the All-Russian Collection of Microorganisms was used.

The strain was maintained and stored using R1 agarized medium of the following composition (g/L): agar, 22.0; soybean flour, 1.0; soluble starch, 10.0; MgSO4, 1.0, K2HPO4, 0.5 (pH 6.8 ± 0.1). For maintenance, the strain was cultured at 29 ºC for 10-12 days with periodical re-inoculations onto a fresh medium. For long-term storage, the culture was freeze-fried and stored in sealed ampoules in a refrigerator.

Vegetation and fermentation media

To obtain seed material, 25-mL tubes containing agar medium with a work culture were washed with 5 mL of sterile physiological solution. The resulted spore suspension was transferred to 500-mL Erlenmeyer flasks containing 100 mL of nutrient medium consisting of the following components (g/L): soybean flour, 21.0; L-lysine, 6.0; yeast extract, 6.0; glucose, 20.0 (pre-sterilization pH 6.8 ± 0.1). The flasks with the seed culture were incubated on a shaker for 46–48 h at 220–240 rpm and 29 ºC. The quality of a seed material was controlled by the microscopic inoculation of diagnostic media (meat-extract agar and Sabouraud glucose agar) and examination of the basic cultivation parameters, such as a pH value at the end of fermentation (should be 6.2–6.8) and the biomass content (no less than 8%).

Ready seed culture (10 vol.%) was aseptically transferred into 500-mL mL Erlenmeyer flasks containing 100 mL of the same nutrient medium and 20g/L of an adsorbing resin added before sterilization. The fermentation was carried out for 214–216 h at 29 ºC and 250 rpm.

Synthetic adsorbing resins

Five different polystyrene/divinylbenzene sorbent types were tested including DIAION™ HP20, HP21, HP 20SS (Mitsubishi Chemicals, Japan), Chromolite and LPS-500 (Technosorbent, Russia). The technical characteristics of the chosen sorbents are shown in Table 1.

Table 1. Basic characteristics of the adsorbing resins included in the study

Sorbent	Matrix type	Surface area, m^2/g	Pore volume, mL/g	Pore size, Å
Chromolite 15AD2	polystyrene/polydivinylbenzene	600–700	1.0–1.4	200–300
DIAION™ HP20	polystyrene/divinylbenzene	590	1.3	290
DIAION™ HP21	polystyrene/divinylbenzene	640	1.3	110
DIAION™ HP 20SS	polystyrene/divinylbenzene	560	1.2	290
LPS-500	polystyrene/polydivinylbenzene	800–900	1.5–1.8	50–500

S. hygroscopicus fermentation in a 15-L fermenter in the presence of a Chromolite 15AD2 resin

The submerged fermentation of the strain was carried out using a 15-L bioreactor manufactured by Prointech Ltd. (Russia) and fermentation medium of the following composition (g/L): soybean flour, 36.2; lysine, 20.4; yeast extract, 5.0; soybean peptone, 5.0; glycerine, 20.0; glucose, 852; NaCl, 5.0; $MgSO_4$, 1.0, $(NH_4)_2SO_4$, 1.0; Na_2HPO_4, 5.0 (pre-sterilization pH 6.8±1). Prior sterilization, the medium was supplemented with the absorbing resin (20 g/L) and Laprol defoamer (1.5 g/L). The steam sterilization was performed in a bioreactor for 1 h at 121–123ºC; then the medium was cooled to 32 ± 1°C; during the cooling process, the air pressure was maintained at the level of 0.03–0.05 MPa by the sterile air delivery into the bioreactor. Both sterilization and cooling processes were carried out at constant stirring (100 rpm). After a completion of the cooling, the medium was aseptically supplemented with sterile 50% glucose solution.

Prior inoculation, the medium was sampled for the microbiological and biochemical control. Then the medium cooled to 28 ± 1°C was inoculated with 1 L of seed material grown in vegetation medium as described above. During fermentation, a constant control and regulation of pH, pO_2, and temperature was performed. The pO_2 level was regulated by changes in the volume of air uptake per a volume of culture broth (manually) and in the rotation speed of a stirring device (automated mode). Culture broth pH was automatically regulated by sterile 20% NaOH solution fed by a peristaltic pump. During the first 48 h, the pH value was maintained within 6.6–6.8.

Starting from 72 h of fermentation, a continuous supply of the bioreactor with 50% sterile glucose solution was carried out in an automated mode with the simultaneous maintenance of the pH and pO_2 values at the earlier determined optimum levels (6.6–6.8 and 30%, respectively).

Determination of a crude biomass content in culture broth

Aliquots of culture broth (10–15 mL) were passed through a stainless steel mesh (hole size <0.1 mm) to separate resin particles. Then the samples were transferred to centrifuge tubes with the known weight and centrifuged at 4500 rpm for 10 min. After a supernatant removal, the tube with mycelial sediment was weighted. The biomass of each sample was determined in three parallel repetitions and calculated using the following formula:

$$X = \left(\frac{b-c}{a-c}\right)\cdot 100\%,$$

where *a* is the weight of a sample-containing tube prior centrifuging (g); *b* is the weight of a sample-containing tube after centrifuging (g); and *c* is the tube weight (g).

Quantification of a rapamycin content in fermentation broth

Rapamycin was extracted from 10-mL samples of fermentation broth (including those containing the sorbent) by their mixing with a double volume of ethyl acetate followed by a 2-h incubation under constant stirring at a room temperature. After a completion of the extraction process, 2 mL of the ethyl acetate extract (or emulsion) was transferred to a new tube and centrifuged at 13400 rpm for 5 min, then 0.5 mL of the upper liquid layer was sampled and evaporated. The resulted solid residue was thoroughly mixed with 0.5 mL of methanol and analyzed by HPLC (see below).

To determine the content of rapamycin absorbed 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 rapamycin extraction and HPLC sample preparation was carried out separately for the resin and filtered fermentation broth as described above.

The HPLC analysis was performed using an Agilent 1200 chromatographic system (Agilent Technologies, USA) with a Zorbax C18-SB column (250 × 4.6 mm, Agilent Technologies, USA) filled with octadecyl silica gel (5 μm). The mobile phase was acetonitrile/distilled water (75/25), the flow rate was 1.0 mL/min at 60°C. The sample volume was 10 μL. The absorbance was measured at 278 nm. A commercial rapamycin preparation (Sigma-Aldrich, USA) dissolved in the methanol was used as the standard. The retention time was 9 min.

Statistical treatment

All fermentations were carried out in three replications. The obtained data were statistically treated using the MS Excel program package.

Results

Effect of adsorbing resins on the rapamycin biosynthesis in flasks

The results of the assessment of the efficiency of adsorbing resin addition in relation to the rapamycin yield are shown in Table 2. According to these data, only DIAION HP20SS and Chromolite 15AD2 demonstrated a positive influence on the strain activity resulting in an increased rapamycin yield comparing to the control. The maximum total rapamycin yield (1330 mg/L) was obtained using the Chromolite 15AD2 resin; it exceeded the yield in the control variant by 10%. Cultivation of *S. hygroscopicus* VKM Ac-2737D in the presence of other resin types resulted in a slight inhibition of the rapamycin production. The minimum yield of the antibiotic (1065 mg/L), which was 12% less than in the control, was observed for the LPS-500 variant.

Table 2. Effect of different adsorbing resins on the rapamycin yield in *Streptomyces hygroscopicus* VKM Ac-2737D

Sorbent	Total rapamycin yield, mg/L	Rapamycin content distribution, mg/L		Sorptive capacity, %
		Sorbent (solid phase)	Culture broth (liquid phase)	
Control (no sorbent)	1210 ± 5	–	–	–
HP20	1190 ± 4	1060 ± 4	130 ± 4	89
HP21	1170 ± 3	1017 ± 3	153 ± 3	87
HP20SS	1271 ± 4	1144 ± 4	127 ± 4	90
Chromolite 15AD2	1330 ± 3	1224 ± 3	106 ± 3	92
LPS-500	1065 ± 4	905 ± 4	160 ± 4	85

In spite of the obtained results, one can note that all tested resins demonstrated a high sorption capacity (85–92%) representing a significant advantage of their use in biotechnological processes.

Assessment of the rapamycin production by *S. hygroscopicus* VKM Ac-2737D in a 15-L bioreactor in the presence of the Chromolite 15AD2 adsorbing resin

To scale the rapamycin production by the *S. hygroscopicus* strain in the presence of the Chromolite 15AD2 adsorbing resin, a 15-L bioreactor with the working volume of 10 L was used. The results of the experiment are shown on Fig. 2. According to them, the active rapamycin biosynthesis started in the beginning of the stationary growth phase (96 h). The maximum antibiotic yield (1420 ± 5 mg/L) was observed after 216 h of fermentation; this result exceeds the earlier obtained level by 11.4% (Dzhavakhiya et al., 2019). Therefore, along with the fermentation medium optimization, the rapamycin yield can be additionally increased by the use of adsorbents, such as the Chromolite 15AD2 resin, during fermentation.

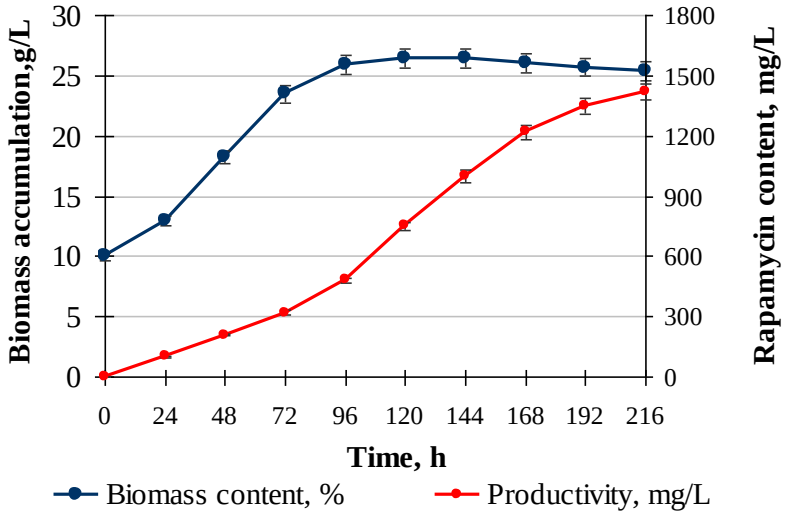

Fig. 2. Fermentation of *S. hygroscopicus* VKM Ac-2737D in a 15-L bioreactor in the presence of the Chromolite 15AD2 adsorbing resin.

Discussion

Biotechnological methods of production of various compounds including antibiotics using metabolic processes in microorganisms represent an efficient tool for the production of valuable biologically active compounds without a need for expensive chemical synthesis. However, there are some limitations to the use of biological methods on an industrial scale (Stark, 2003). One of such factors is the self-inhibition of the biosynthetic activity of microorganisms by the generated products. To overcome the cytotoxic action of the produced compound, one can *in situ* remove it from the reaction medium (Bechtold, 2009; Phillips, 2013). To maximally use the advantages of this approach, highly selective removal of a target compound on an adsorbing phase is required (Bechtold, 2009). However, as it has been already mentioned earlier, the use of synthetic adsorbing

resins is not a universal method, and the suitable resin type should be determined for each biosynthetic process, since even resins with similar physical characteristics may provide either increase or reduction of the yield of a target compound in culture broth (Lam, 1995).

In our case, we tested five polystyrene/divinylbenzene adsorbing resins with similar characteristics (Table 1). DIAION HP20 and DIAION HP21C resins are highly cross-linked polymers with spherical particles characterized by a porous structure and the surface area within 500–1200 m²/g of dry matter. They are used for adsorption refining of peptides, proteins, polyphenol compounds, cephalosporin C, and for the chromatographic separation and purification of fermentation products (Kamiyama, 1994). DIAION HP20SS developed based on DIAION HP20 is characterized by small-size particles and controlled pore size distribution and is used for separation and purification of a wide range of molecules, from small peptides and oligonucleotides to large proteins (Rani, 2020). Chromolite 15AD2 is a macroporous adsorbent used for the reverse-phase chromatography of organic compounds and can successfully replace hydrophobic silica gel sorbents of the C8 and C18 type. It works at any pH values of eluents and regenerating solutions and is characterized by a rigid polymeric matrix and a uniform particle size that provides a high separation efficiency. LPS-500 is a highly cross-linked polystyrene sorbent characterized by a rigid matrix of the optimal porosity and used for the sorption refining of flavonoids, peptides, proteins, drug substances, and radioactive isotopes.

There are some examples of a successful application of some of the above-mentioned adsorbing resins in the processes of antibiotic biosynthesis. The use of the DIAION HP20 resin made it possible to remove the toxic action of the teicoplanin antibiotic on the growth of its producer, *Actinoplanes teicomyceticus*, which resulted in a 4.2-fold increase of its final titer and reduced the number of required extraction stages (Lee, 2003). A similar positive effect was reported for the use of DIAION HP20 in the biosynthesis of thailandepsin A, a promising antitumor agent produced by *Burkholderia thailandensis* E264 (Liu, 2012). The use of the DIAION HP21 resin in the process of fermentation of *Streptomyces achromogenes v. rubradiris* provided a fourfold increase in the rubradirin yield (Marshall, 1990).

A comparative analysis of the effect of the chosen adsorbents on the final rapamycin yield showed that only two adsorbing resins, HP20SS and Chromolite 15AD2, provided a 5% (1271 mg/L) and 10% (1330 mg/L) increase of this parameter, respectively, comparing to the control. These sorbents added to the fermentation medium before fermentation can a highly selective (90 and 92%, respectively) sorption of the target antibiotic from the culture broth that provides a significant simplification of the rapamycin extraction scheme. Moreover, the sorbents provide almost complete removal of related compounds (minor components, pigments, protein products, salts, etc.), which remain in the culture broth. Other testing resins (DIAION HP20, DIAION HP21, and LPS-500) showed slightly reduced rapamycin yield. These results can be explained base on the features of sorbent particles (Table 1). For example, the pore size in LPS-500 resin, which showed the worst result, is rather heterogeneous and reaches 500Å that exceeds the corresponding parameter of other adsorbents. Since all resin types were added to the fermentation medium before fermentation, this resin could absorb any medium components or metabolites important for the rapamycin biosynthesis; their deficiency, in turn, could result in the reduction of the final antibiotic concentration. To clarify the reasons for the observed phenomenon, further investigations are needed.

The main advantages of the Chromolite 15AD2 resin are the relatively low cost, excellent physical and chemical stability, thermal stability, and high exchange capacity. The resistance of this sorbent to organic solvents makes it reusable for the rapamycin production process. In this study, this resin was successfully used for the scaling of the rapamycin biosynthesis for a 15-L bioreactor providing an 11.4% increase in the final yield of this antibiotic comparing to the earlier obtained results (Dzhavakhiya et al., 2019). Thus, the result of this study may be of certain interest for the further scaling up of the process of industrial rapamycin production by *S. hygroscopicus*.

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