Optimization of the 9α-hydroxylation of steroid substrates using an original culture of Rhodococcus erythropolis

V. A. Andryushina, N. V. Karpova, T. S. Stytsenko, V. V. Yaderets, E. D. Voskresenskaya, V. V. Dzhavakhia

Federal Research Center “Fundamentals of Biotechnology”, Russian Academy of Sciences, Moscow, Russian Federation

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Introduction
Deterioration of environmental conditions and the corresponding increase in the number of allergic and inflammatory diseases on the global scale gives rise to a growing need for steroid preparations of different therapeutic action. According to analytic forecasts, the volume of the steroid market in 2021 will reach 4.480 million US dollars (Fernandez-Cabezón et al., 2018; Barredo & Herráiz, 2017; Fernandes et al., 2003). Thus, despite the existing achievements in the biotechnology of steroid production, the development of effective methods of synthesis of androgenic, gestagenic, mineral, and glucocorticoid steroid preparations from natural plant products still remains relevant (Brzżinska et al., 2013; Guevara et al., 2017; Fernandez-Cabezón et al., 2018).

The main indications for glucocorticoid therapy of humans include rheumatoid arthritis, bronchial asthma, neurodermatitis and other skin and allergic diseases, acute adrenal insufficiency, and also various shock states (post-rheumatic, operational, toxic, burn, etc.). Today the most common anti-inflammatory steroids represent structural modifications of natural compounds and are characterized by improved therapeutic properties and weaker side effects. Among such preparations, fluorinated corticosteroids (dexamethasone, sinatilin, triamcinolone, fluticasone, etc.) are in the greatest demand (Mashkovskiy, 2012). 9α-hydroxy-4-ene-3,17-dione (9α-OH-AD) is the basic intermediate in the synthesis of the aforementioned drug preparations from sterols (Fernández de las Heras et al., 2012; Mohin et al., 2012; Jakóbczans et al., 2016; Mondaca et al., 2017). According to the existing data, 9α-hydroxylation, which represents a key reaction in the synthesis of fluorinated corticosteroids, cannot be realized via chemical synthesis (Guevara et al., 2017). Therefore, a cost-efficient large-scale production of this class of drugs is possible only with the use of microorganisms able to perform a highly selective targeted transformation of steroid molecules of a given structure under ecologically safe conditions and without any need for preliminary protection of the functional groups of the initial molecule (Mutafiova et al., 2016; Smirna et al., 2017).

To obtain inoculation material (cultivation stage 1), the biomass of Rhodococcus erythropolis VKPM AC-1740 was transferred from agar slants into 750 ml conic flasks containing 100 ml of vegetation media of the following composition (g/l): medium 1 – yeast extract, 10.0; glucose, 10.0; soybean flour, 10.0; KH₂PO₄, 2.0; Na₂HPO₄, 4.0 (pH 6.8–7.4); medium 2 – corn extract, 15.0; glucose, 10.0; KH₂PO₄, 2.0; Na₂HPO₄, 4.0 (pH 6.8–7.4). The culture was grown on a rotary shaker (220 rpm) for 68–72 h at 28–29 ºC. To obtain a working biomass (cultivation stage 2), the inoculum obtained at the stage 1 was transferred into flasks containing the same media (the volume of seed material was 20% of the medium volume) and grown under the same conditions for 23–25 h. During a study of the effect of the inducer concentration on the rate of 9α-OH-AD formation, different concentrations (0.25, 0.50, and 1 g/l) of the AD solution in dimethylformamide (DMF) were added to the vegetation medium after 6 h of incubation. To perform AD transformation at a load of 5 g/l, 10 ml of Rh. erythropolis cells at the age of 23–25 h were transferred into 750 mL flasks with baffles containing 40 mL of vegetation medium supplemented with the steroid. AD was added in the form of microcrystals or suspension with a surfactant or DMF. The process was carried out at 28–29 ºC and with constant mixing (220 rpm). During AD transformation at a load of 10–30 g/l, the steroid was preliminarily precipitated from DMF solution. The resulting paste was mixed with a surfactant and transformation medium. The obtained homogeneous suspension was poured in equal amounts into the flasks with baffles, and then a concentrated cell mass was added (25 vol.%). To obtain a cell concentrate, cells were centrifuged for 1 h at 1500 rpm at the age of 23–25 h. The resulting biomass was homogenized, supplemented with a fresh medium to the required volume, and added into transformation flasks. The amount of a biomass required for AD transformation at a load of 10 g/l was 3.13 g/l (dry weight); in the case of a 30 g/l load, the biomass was added by two equal portions, and its total amount was 6.2 g/l (dry weight). The amount of 9α-OH-AD in a culture broth was evaluated by a thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Steroids were extracted by ethylacetate. To perform TLC, Sorbifil plates (Russia) and benzol: acetone mix (3:1) were used. HPLC was performed on a Gilson chromatographer (United States) equipped with a Silarorb C-18 column (10 μm, 4.0 x 250 mm); the flow rate was 0.8 ml/min. The mobile phase was MeOH: H₂O mix (70:30). The absorbance was measured at 260 nm. Replacement of corn extract, which has an unstable composition, by yeast extract and soybean flour and the use of glucose as an optimal carbon source for a Rh. erythropolis culture have provided a high-yield production of 9α-hydroxy-4-ene-3,17-dione with increased AD loads. Use of such techniques as the inoculum induction and application of surfactants have provided a positive effect on the AD transformation with a load exceeding 10 g/l. During 9α-hydroxylation of AD with a load of 30 g/l, a target product with the yield of 83% has been obtained.

Keywords: fluorinated corticoids; AD; 9α-OH-AD; Rhodococcus erythropolis VKPM AC-1740; surfactants; inducer.
It is known that some lower fungi (Ascomycota, Helicostomata, Cercinella) and many bacteria, especially actinobacteria (Arthrobacter, Corynebacterium, Mycobacterium, Nocardia, Rhodococcus) are able to introduce 9α-hydroxy groups into a steroid molecule. However, steroid hydroxylation with fungi does not provide a sufficient selectivity of the process. Along with the formation of a target hydroxysteroid, fungi also produce a number of side mono- and dihydroxy-products, which complicates the further isolation of the target product and significantly reduces its yield. In addition, both position and orientation of an introduced hydroxyl group strongly depends on the structure of the steroid molecule (Donova & Egorova, 2012; Andryushina et al., 2013; Barreto & Herráiz, 2017).

Unlike fungi, bacteria perform 9α-hydroxylation reaction regardless of the steroid structure and presence of additional bonds and substituents. Therefore, actinobacteria capable of providing a high-selective introduction of a hydroxyl group at the 9α-position of a steroid molecule may be considered as the most promising bioreagents, since the corresponding bacterial enzyme shows the lower substrate specificity than steroid 9α-monoxygenase of a fungal origin (Petrunsa et al., 2009, 2011; Lee et al., 2016; Nielsen & Keasling, 2016). However, 9α-hydroxylation activity was detected only in bacteria which were able to use steroids as a carbon source since this reaction represents an intermediate stage of a complete cleavage of steroid molecules to CO2 and H2O, at which the simultaneous action of 3-ketosteroid-1,2-dehydrogenase and 9α-hydroxylase is observed (Petrunsa et al., 2014; Donova & Egorova, 2012; Rodina et al., 2009). The analysis of published data showed that the selective 9α-hydroxylation without any destruction of a steroid nucleus can be performed using bacterial strains carrying mutations, which block the biosynthesis of 1,2-dehydrogenase or prevent the functioning of this enzyme. In our previous study, we obtained a highly-selective Rhodococcus erythropolis strain VKPM AC-1740 with improved 9α-hydroxylation activity (Rodina et al., 2009; Carpova-Rodina et al., 2011). Using this strain, we developed efficient technologies for the production of 9α-hydroxy-stereoid derivatives (Vojshvillo et al., 2007) and developed several efficient methods for 9α-OH-AD production from steroids including the mixed culture method (Andryushina et al., 2011) or the use of original biocatalyst representing R. erythropolis VKPM AC-1740 cells immobilized in a PVA cryogel (Carpova-Rodina et al., 2011).

A common disadvantage of all methods for 9α-hydroxylation of steroids, which were previously developed by our team, was the dependence of the hydroxylation activity of R. erythropolis VKPM AC-1740 on a nutrient medium composition, namely, the quality of the corn extract used as the main nitrogen source (Ribeiro et al., 2017; van der Geize et al., 2001, 2001, 2008). The composition of a corn extract is determined by several factors, such as the grain quality, scheme and mode of its processing. According to the obtained data, the best result was obtained using a modified medium containing soybean flour, yeast extract, and glucose (Rodina et al., 2009; Carpova-Rodina et al., 2011). Using this strain, we introduced a new source of organic nitrogen – a culture grown on such medium, there is a need to select an alternative method for 9α-OH-AD as an individual compound. Therefore, this medium was chosen as a stable and available source of organic nitrogen.

The earlier developed R. erythropolis VKPM AC-1740 strain characterized by a high 9α-hydroxylation activity in relation to Δ4-3-ketosteroids, was used in the study. The culture was stored on solid agar medium of the following composition (g/l): yeast extract, 10.0; glucose, 10.0; KH2PO4, 1.0 (pH 6.8–7.2). To obtain inoculation material (cultivation stage 1), the biomass of R. erythropolis was transferred from agar slants into 750 ml conic flasks containing 100 ml of vegetation media of the following composition (g/l): medium 1 – yeast extract, 10.0; glucose, 10.0; soybean flour, 10.0; KH2PO4, 2.0; Na2HPO4, 4.0 (pH 6.8–7.4); medium 2 – corn extract, 15.0; glucose, 10.0; KH2PO4, 2.0; Na2HPO4, 4.0 (pH 6.8–7.4). The culture was grown on a rotary shaker (220 rpm) for 68–72 h at 28–29 °C.

To obtain a working biomass (cultivation stage 2), the inoculum obtained at the stage 1 was transferred into flasks containing the same media (the volume of seed material was 20% of the medium volume) and grown under the same conditions for 23–25 h. During the study of the effect of the inducer concentration on the rate of 9α-OH-AD formation, different concentrations (0.25, 0.50, and 1 g/l) of the AD solution in dimethyloxamidam (DMF) were added to the vegetation medium after 6 h of incubation.

**Biotransformation conditions.** To perform AD transformation at a load of 5 g/l, 10 ml of R. erythropolis cells at the age of 23–25 h were transferred into 750 ml flasks with baffles containing 40 ml of vegetation medium supplemented with the steroid. AD was added in the form of microcrystals or suspension with a surfactant or DMF. The process was carried out at 26–29 °C and a constant mixing (220 rpm).

During AD transformation at a load of 10–30 g/l, the steroid was preliminarily precipitated from the DMF solution. The resulting paste was mixed with a surfactant and transformation medium. The obtained homogeneous suspension was poured in equal amounts into the flasks with baffles, and then a concentrated cell mass was added (25 vol.%).

To obtain a cell concentrate, cells were centrifuged for 1 h at 1500 rpm at the age of 23–25 h. The resulting biomass was homogenized, supplemented with a fresh medium to the required volume, and added into transformation flasks. The amount of a biomass required for AD transformation at a load of 10 g/l was 3.13 g/l (dry weight); in the case of a 30 g/l load, the biomass was added by two equal portions, and its total amount was 6.2 g/l (dry weight).

**Transformation efficiency assessment.** The amount of 9α-OH-AD in a culture broth was evaluated by a thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Steroids were extracted by ethylacetate. To perform TLC, Sorbifil plates (Russia) and benzol: acetone mix (3 : 1) were used. HPLC was performed on a Gilson chromatograph (United States) equipped with a Silasor C-18 column (10 μm, 4.0 × 250 mm); the flow rate was 0.8 ml/min. The mobile phase was MeOH: H2O mix (70 : 30). The absorbance was measured at 260 nm.

**Results**

**Selection of Nitrogen and Carbon Sources.** At the first stage of a nutrient medium optimization, a comparative 9α-hydroxylation of AD with a 5 g/l load was carried out using media 1 and 2. The obtained results were identical for both media: a complete conversion of the initial compound with the formation of 4.7–4.8 g/l of 9α-OH-AD occurred within 20–22 h. Replacement of a carbon source in the medium 1 provided a more significant impact on the AD biotransformation process (Table 1). According to the obtained data, the best result was obtained using a modified medium containing soybean flour, yeast extract, and glucose. After 21 h of the AD transformation (5 g/l load) in a modified medium, almost complete substrate conversion was observed with the formation of 9α-OH-AD as an individual compound. Therefore, this medium was chosen as the basic one for the further optimization of the conversion process.

**Table 1**

| Carbon source | Average steroid content in a culture broth, % |
|---------------|---------------------------------------------|
| (5 g/l)       |                                             |
| Control (no carbon source) | 68 ± 1.5 | 30 ± 1.5 |
| Glucose       | 98 ± 1.0                                     | 2 ± 1.0  |
| Fructose      | 15 ± 1.3                                     | 84 ± 1.3 |
| Sucrose       | 40 ± 0.8                                     | 60 ± 0.8 |
| Lactose       | 12 ± 1.5                                     | 85 ± 1.5 |

*Note:* hereinafter, the experimental error (±) was determined as a random error of direct measurements using the following formula: Δx = (xmax − xmin)/2, where xmax and xmin are the maximum and minimum values obtained by the series of repeated measurements.

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Effect of the AD introduction method on the 9α-OH-AD output. As we have mentioned earlier, one of the main problems of microbiological transformations of steroid substrates is the high hydrophobic property of steroid molecules; the solubility of steroids belonging to the androstane group does not exceed 20 mg/l (Goetschel & Bar, 1992). To solve this problem, we added AD into a medium either in the form of a fine-dispersed suspension with various surfactants, or dissolved in DMF (Table 2).

Table 2  
Effect of various surfactants on the production of 9α-OH-AD by Rhodococcus erythropolis VKPM AC-1740

| Surfactant used for introduction of ground AD into fermentation medium | Average steroid content in a culture broth (TLC), % | 9α-OH-AD | AD |
|---|---|---|---|
| Tween 21* | 97 ± 1.5 | 3 ± 1.5 |
| Tween 40 | 96 ± 1.3 | 4 ± 1.3 |
| Tween 61 | 96 ± 1.4 | 4 ± 1.4 |
| Tween 80 | 99 ± 0.3 | 1 ± 0.3 |
| Triton 100 | 95 ± 1.6 | 5 ± 1.6 |
| Span 20 | 98 ± 1.3 | 2 ± 1.3 |
| Span 80 | 97 ± 1.5 | 3 ± 1.5 |
| Emululan EL | 90 ± 1.3 | 10 ± 1.3 |
| DMF** | 95 ± 1.3 | 5 ± 1.3 |
| Control | 98 ± 0.5 | 2 ± 0.5 |

Note: * – surfactant; AD ratio is 1 : 4; ** – DMF 4 vol.%.

According to the obtained results, the most efficient AD transformation at a 5 g/l load was observed in the variant with Tween 80. However, the control variant (i.e., without addition of any surfactant or solvent) demonstrated a quite comparable result.

Effect of the Inducer on a Microbiological 9α-Hydroxylation by R. erythropolis VKPM AC-1740. 3-Ketosteroid-9α-hydroxylase (9-KSH) is a two-component non-heme iron-dependent Rieske monoxygenase consisting of a KshA oxygenase and a KshB reductase. 9-KSH is one of the key enzymes involved into a microbiological degradation of steroids and is of a great physiological importance for a wide range of steroid-transforming bacteria (Petrusma et al., 2014). The presence of a 3-keto group and also Δ1 and / or Δ4-double bonds in the A ring of the steroid nucleus is a necessary condition for a 9α-hydroxylation activity (Akhem & Titov, 1970).

To study the stimulation of a 9-KSH biosynthesis, the inoculum of R. erythropolis VKPM AC-1740 was grown on a medium supplemented with AD as an inducer (0.25, 0.50, and 1.0 g/l). The AD solution was added 6–7 h after inoculation by a second-generation culture. The total time of incubation was 23–25 h. The amount of the inoculum used for transformation of AD (5 g/l) was 20% of the medium volume. The result of the experiment is shown in Table 3.

Table 3  
Consumption of AD (5 g/l) and accumulation of 9α-OH-AD by R. erythropolis VKPM AC-1740 grown in the presence of various concentrations of the inducer

| Time, h | Average steroid content in a culture broth, % | 9α-OH-AD | AD |
|---|---|---|---|
| | control | 0.25 | 0.50 | 1.00 |
| AD | AD | AD | AD | AD |
| 0 | 5.0 | 5.0 | 5.0 | 5.0 |
| 2 | 5.0 | 4.9 | 0.1 | 4.8 | 0.1 | 4.7 | 0.2 |
| 4 | 5.0 | 4.7 | 0.2 | 4.6 | 0.2 | 4.5 | 0.3 |
| 6 | 4.8 | 0.1 | 4.4 | 0.3 | 4.4 | 0.3 | 4.3 | 0.4 |
| 12 | 1.9 | 2.9 | 1.5 | 3.2 | 1.3 | 3.4 | 1.2 | 3.3 |
| 14 | 1.4 | 3.5 | 0.9 | 3.1 | 0.9 | 3.3 | 0.8 | 3.0 |
| 16 | 1.0 | 3.9 | 0.4 | 3.0 | 0.3 | 3.1 | 0.2 | 2.9 |
| 18 | 0.6 | 4.3 | – | 2.5 | – | 2.6 | – | 2.5 |
| 20 | 0.1 | 4.7 | – | 2.1 | – | 2.4 | – | 2.0 |

The 9α-hydroxylating activity of R. erythropolis VKPM AC-1740 in the presence of the inducer was observed already after 2 h of transformation, whereas in the control variant it was registered only after 6 hours of transformation. For all variants with induced seed material, we observed an active destruction of the target product during transformation. A probable explanation is that the induction of a 9-KSH biosynthesis may result in the induction of 1,2-dehydrogenase, responsible for the next reaction in the steroid nucleus degradation pathway.

Results obtained in this experiment were used for the study of the AD transformation at a load of 10–30 g/l. AD transformation into 9α-OH-AD at a substrate load of 10 g/l. The effect of induction on the AD transformation at a load of 10 g/l was examined using a concentrated R. erythropolis VKPM AC-1740 biomass (3.13 g/l of dry weight). The transformation time was 19–20 h. The yield of 9α-OH-AD equal to 94.3% and 94.0% (as determined by HPLC) was obtained in the case of the inducer concentration equal to 0.5 g/l; this yield exceeded that of the control by 10% (Fig. 1). A slight destruction of the target product was observed at the inducer concentration equal to 1.0 g/l (Figs. 1, 2).
formation process, an additional portion of a concentrated inoculum and a glucose solution (5 g/l) were added during the process (Fig. 3).

According to this experiment, the substrate was completely transformed into 9α-hydroxy product within 44 h of incubation. The yield of a technical 9α-OH-AD substance reached 83%, while its content determined by HPLC was 93.6%.

**Discussion**

According to many authors, nutrient medium composition influences the direction of the steroid reaction and on the rates of consumption of the initial substrate and accumulation of the target product. For example, changes in the concentration or the origin of organic nitrogen in a nutrient medium directly affect the biomass accumulation rate and can change the hydroxylation activity of cells. It was shown that the use of non-dehydrated nitrogen sources, such as casein, gelatin, and meat extract, provided a biomass possessing the higher transformational activity (Angelova et al., 1995). Soybean flour contains fatty acids, which positively influence the permeability of a bacterial cell wall and improve the availability of lipophilic substrates, such as steroid compounds, for enzymatic influence the permeability of a bacterial cell wall and improve the availability of lipophilic substrates, such as steroid compounds, for enzymatic influence the permeability of a bacterial cell wall and improve the availability of lipophilic substrates, such as steroid compounds, for enzymatic

The results obtained in our study revealed no correlation between the biotransformational ability of *Rh. erythropolis* VKPM AC-1740 and the nitrogen source. The effect of a carbon source on the yield of a target 9α-OH-AD was more significant. We showed that the absence of glucose (preferred carbon source) in the medium resulted in a decreased 9α-hydroxylation rate as compared with the glucose-containing medium (Table 1). We also observed the inhibition of a 9α-hydroxylase activity in the media containing lactose or fructose as a carbon source. Our results agree with the data obtained by Angelova et al. (1995), who also showed the maximum biomass accumulation and a high substrate conversion rate in a glucose-containing medium.

A high hydrophobic property of steroid substrates in aqueous media results in a low degree of conversion of the initial substrate and a low yield of the target reaction product. Traditional approaches to reducing mass transfer limitations include the use of a fine-grain substrate and various surfactants or solvents able to mix with water, such as dimethyl sulfoxide, DMF, methanol, acetone, and 1,2-propanediol. To prevent deactivation of the biocatalyst, the amount of the added solvent usually does not exceed 1.5–5.0% (v/v) (Fernandes et al., 2003). In addition, it is known that solvents may provide different effects, such as changes in the rate and direction of hydroxylation, as well as the stimulation or suppression of the by-product formation. According to some authors, microorganisms can include some solvents into their metabolic pathways to regenerate reduced cofactors (Angelova et al., 1995). Avramova et al. (2010) showed that the presence of Tween 80 in a glucose-containing medium accelerated the process of the 9α-hydroxylation of AD.

In this study we showed that the surfactant presence in the nutrient medium during the AD transformation at a load within 5 g/l did not result in an expected increase in the 9α-OH-AD generation rate as compared to the control (Table 2). A probable reason is that the strain itself may be able to emulsify and degrade hydrophobic substrates. According to the existing publications, such ability is determined mainly by specific structural features of the cell membrane of this microorganism, which is lipophilic, i.e., has a high affinity to hydrophobic substrates (Tsitsko et al., 1999). The surface activity and hydrophobic character of the cell membrane promote interaction between the cells and insoluble substrate, which makes it possible to overcome a limited diffusion during the substrate uptake into a cell (Kostina, 2008). In addition, the composition of soybean flour includes lecithin, a natural emulsifier preventing aggregation of steroid particles. Soybean flour also contains lecithin, a natural emulsifier preventing aggregation of steroid particles. Soybean flour also contains lecithin, a natural emulsifier preventing aggregation of steroid particles.

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**Fig. 3.** Dynamics of 9α-OH-AD accumulation at the AD load of 30 g/l

According to this experiment, the substrate was completely transformed into 9α-hydroxy product within 44 h of incubation. The yield of a technical 9α-OH-AD substance reached 83%, while its content determined by HPLC was 93.6%.

**Conclusion**

Replacement of corn extract with yeast extract and soybean flour, as well as the choice of glucose as a carbon source provided reduction of the duration of transformation while still maintaining a high 9α-OH-AD yield. At the same time, the effect of surfactants and the effect of 9-KSH-mediated induction of *R. erythropolis* VKPM AC-1740 were positive only if they were applied for transformation with an increased substrate load. A 44-h AD transformation at a load of 30 g/l by *R. erythropolis* VKPM AC-1740 resulted in the production of 9α-OH-AD with the yield of 83.0%; the content of the target compound determined by HPLC was 93.6%.

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