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Short Communication

Enhanced cell surface hydrophobicity favors the 9 α -hydroxylation of androstenedione by resting *Rhodococcus* sp. cells

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The achievement of an effective process of 9 α -hydroxylation of 4-androstene-3,17-dione is of significant importance as it leads to the formation of the key intermediate 9 α -hydroxy-4-androstene-3,17-dione which is not possible by chemical means. In this study, the 9 α -hydroxylation of 4-androstene-3,17-dione was carried out by resting *Rhodococcus* sp. cells. The ability of the naturally hydrophobic *Rhodococcus* to assimilate *n*-alkanes was employed to obtain a cell depot with an intentionally increased cell surface hydrophobicity. The control *Rhodococcus* sp. cells were cultivated on medium containing glucose instead of *n*-alkanes as a source of carbon and energy. Cells were harvested, washed from the cultivation media, and subjected to transformation of crystal androstenedione in buffer medium. The hydrophobicity of the *n*-alkanes- and glucose-grown cells, their total lipid content, and fatty acid composition were determined. The ultrastructure of the *n*-alkanes- and glucose-grown cells and their steroid hydroxylating activities were examined and compared. The results obtained in the present study showed that the intentionally achieved growth-driven enhancement of the already hydrophobic *Rhodococcus* sp. cells made them even more compatible with the hydrophobic steroid substrate and enhanced its accessibility, which provided an increased steroid hydroxylating activity and lack of the accompanying product destruction.

Keywords: 9 α -steroid hydroxylation / Androstenedione / Hydrophobicity / *Rhodococcus* / Ultrastructure

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1 Introduction

The achievement of an effective process of microbial 9 α -steroid hydroxylation is in the focus of interest of pharmaceutical industry as an essential step of the steroid drugs manufacturing allowing for a shorter way to the synthesis of fluorinated corticosteroids [1].

There are different approaches applied for achievement of an effective microbial transformation of steroid compounds [2]. Most of them attempt to overcome the main bottlenecks in

the process performance, either the low water solubility of the steroid substrate or the destruction of the desired product, or both. In our previous studies of the 9 α -hydroxylation of AD by resting *Rhodococcus* sp. cells we dissolved AD in water miscible organic solvents [3], applied it as water suspension obtained by ultrasonification [4], used non-ionic surfactants as mediators of substrate solubility [5], aqueous-organic solvent two-phase systems as well as organic solvent as a sole component of the reaction medium [6]. Studying inducibility of the *Rhodococcus* sp. 9 α -steroid hydroxylase we managed to avoid the destruction of the 9 α -OH-AD and demonstrated almost complete transformation of AD into the desired product [7]. Based on all our previous studies we are inclined to make the conclusion that the achievement of robust cell-substrate contact is a significant factor allowing for increasing of their 9 α -steroid hydroxylating activity.

The present investigation is a continuation of our experiments to find conditions for an effective 9 α -hydroxylation of AD with resting *Rhodococcus* sp. cells. The hypothesis was that the preparation of a cell depot of *Rhodococcus* sp. cells

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Abbreviations: 9 α -OH-AD, 9 α -hydroxy-4-androstene-3,17-dione; 9 α -OH-ADD, 9 α -hydroxy-4-androstadiene-3,17-dione; AD, androstenedione; 4-androstene-3,17-dione; ADD, 4-androstadiene-3,17-dione; *isom*, isomeric; Me, branched chain; SEM, Scanning electron microscopy; TEM, Transmission electron microscopy

with an additionally increased hydrophobicity and application of a crystal AD would favor the effectiveness of the 9α -steroid hydroxylation reaction. To enhance hydrophobicity, we cultivated the *Rhodococcus* sp. cells on media containing *n*-alkanes as a sole source of carbon and energy while control cells were cultivated on glucose. The hydrophobicity, the total lipid content, and fatty acid composition of the *n*-alkanes- and glucose-grown cells were determined. Their ultrastructure and steroid hydroxylating activities were examined and compared.

2 Materials and methods

The *Rhodococcus* sp. strain, medium and inoculum preparations, and cultivation conditions were described previously [4]. *n*-Alkanes, 2% (v/v), were added into the cultivation medium instead of glucose before its inoculation. The *n*-alkanes mixture contained the following hydrocarbons: C9 - 0.27%, C10 - 1.21%, C11 - 22.8%, C12 - 37%, C13 - 25.4%, C14 - 0.22%, C15 - 0.79%, C16 - 0.4%, C17+C18 <0.1%, and traces of C19+C20.

Transformation of AD, analytical procedures, SEM and TEM procedures were described previously [5]. The cell surface hydrophobicity was determined by microbial cell adhesion to hydrocarbons [8]. The crystal AD was added in reaction medium in concentration 1 mg/mL.

The fatty acid composition was determined by gas chromatography-mass spectrometry after extraction and transmethylation of the total lipids according to Cristie [9]. An Agilent 6890 Plus System with 5973 mass selective detector and HP-5MS capillary column, 60 m $\times$ 0.25 mm $\times$ 0.25 μ m (Agilent Technologies, USA) were used. Helium was the carrier gas at a flow rate of 0.8 mL/min. Hydrocarbons were analysed by temperature gradient started from 80°C with 20 K/min to 150°C, then with 5 K/min to 320°C and held at this temperature for 15 min. Fatty acids methyl esters were measured by temperature gradient started from 150°C with 2 K/min to 210°C, then with 5 K/min to 280°C and 30 min held at that temperature. T_{inj} was 280°C and T_{aux} 300°C. Injection volume was 1 μ L; split 20:1. Peaks identification was according to mass spectral library.

All numeric data presented are mean values of three independent experiments.

3 Results and discussion

The cell surface hydrophobicity of *n*-alkanes-grown cells was determined as 100%, of glucose-grown ones it was 72%. The total amount of cell lipids was 63 mg/g in *n*-alkanes-grown cells and 11 mg/g in glucose-grown ones. The same fatty acids are found in the cells, irrespectively of the carbon and energy source, albeit at different ratios (Table 1). The total amount of the saturated and unsaturated fatty acids and the isomeric forms are almost equal. The amount of the branched chain fatty acids, however, is 3.6 times higher in glucose-grown cells. The even number fatty acids are better presented in glucose-grown cells with the exception of

Table 1. Fatty acid composition of *Rhodococcus* sp. glucose- and *n*-alkanes-grown cells

Fatty acid	In glucose-grown cells, %	In <i>n</i> -alkanes-grown cells, %
9:0	Traces	0.4
10:0	0.1	1.0
11:0	Traces	0.9
12:0	0.4	2.5
13:0	0.2	13.7
14:0	5.2	4.9
14:1	0.3	0.1
15:0	3.2	5.7
15:1	0.4	0.4
16:0	16.7	9.2
Me-16:0	0.1	0.1
isom. 16:1	11.9	4.1
Me-16:1	0.1	0.1
17:0	1.7	3.2
Me-17:0	3.0	2.2
isom. 17:1	9.1	6.7
18:0	2.5	3.1
Me-18:0	19.4	3.7
isom. 18:1	13.6	11.9
18:2	1.0	1.8
19:0	0.6	0.5
Me-19:0	0.8	0.4
isom. 19:1	3.4	13.5
20:0	0.5	1.0
isom. 20:1	3.6	4.1
21:0	0.1	0.6
isom. 21:1	0.2	1.3
22:0	0.8	0.5
isom. 22:1	0.4	0.8
23:0	0.1	0.4
isom. 23:1	Traces	0.6
24:0	0.3	0.3
isom. 24:1	0.2	0.2
25:0	Traces	0.1
isom. 25:1	Traces	0.1
26:0	Traces	Traces
isom. 26:1	0.1	Traces

12:0, which is about six times more in the *n*-alkanes-grown ones. Odd number fatty acids in glucose-grown cells are almost twice the amount in *n*-alkanes-grown ones with the most significant differences observed in 13:0 and isomeric 19:1.

It is worth to relate the length of fatty acids detected in the *n*-alkanes-grown *Rhodococcus* sp. cells to the chain length of the hydrocarbons in the *n*-alkanes mixture used as a source of carbon and energy in the cultivation medium. Thus, the hydrocarbons with 10 to 13 C atoms dominate in the *n*-alkanes mixture and represent 86.4% of all hydrocarbons. The fatty acids with 10 to 13 C atoms in their chains represent 18.1% in the *n*-alkanes-grown cells and 0.7% of the whole fatty acids in glucose-grown cells ones. This means that the amount of C10-C13 fatty acids in *n*-alkanes-grown cells is 26 times higher compared to glucose-grown ones. This observation is in a good correlation with the earlier reports that the hydrocarbon-utilizing bacteria contained considerable amounts of fatty acids with chain lengths

Figure 1. SEM of glucose- (A, B) and *n*-alkanes-grown (C, D) *Rhodococcus* sp. cells. Bar = 0.1 μm .

equivalent to those of the hydrocarbon substrate [10]. Even long-chained haloalkanes are incorporated into the cellular fatty acids by *Rhodococcus rhodochrous* cells [11].

As seen on SEM micrographs, the glucose-grown cells (Fig. 1A and 1B) are with a dense surface and distinct even outlines. They are covered by an amorphous material, which is amassed on certain surface zones. Somewhere a separation of the amorphous material from the surface is also observed. The *n*-alkanes-grown cells (Fig. 1C and 1D) are with a reduced turgor. They are deformed and with curved uneven surfaces. The cells' outlines are not smooth and some concave sites appeared on them. At some places, the surface membranes are disrupted.

TEM investigations revealed some more differences in the ultrastructure of the *Rhodococcus* sp. cells. Cell surface membranes of glucose-grown cells are fitted closely to the cytoplasm and an electron dense material is found to cover them (Fig. 2A and 2B). In few of the cells are seen small transparent zones located in the nucleoid area (Fig. 2A). Fibrillar structures (Fig. 2B) similar to those in *Mycobacterium* cells [12] are observed in some of the cells. A peculiar characteristic of the *Rhodococcus* sp. cells grown on *n*-alkanes (Fig. 3A, B and C) is the presence of large electron-transparent inclusion bodies in most of the cells. They are located in the nucleoid regions (Fig. 3A) as well as closely to the disrupted cell surface membranes (Fig. 3B) and in the area of mesosome (Fig. 3C). Our results are in correlation with reported earlier observations that the cultivation on hydrocarbons as a sole source of carbon and energy leads to appearance of large electron-transparent inclusion bodies in *Rhodococcus*

opacus [13] as well as in changes in the ultrastructure of *Rhodococcus erythropolis* [14].

Figure 4 presents the dynamics of the 9α -steroid hydroxylation reaction carried out by resting glucose- and *n*-alkanes-grown *Rhodococcus* sp. cells. As seen from the curves, the two types of cells reach the maximum in the product accumulation for about 30 h. There is a lag-phase in the curve of 9α -OH-AD accumulation by glucose-grown cells and the amount of the accumulated 9α -OH-AD is twice as low as the one accumulated by *n*-alkanes-grown *Rhodococcus* sp. cells. After reaching the peak, the amount of the 9α -OH-AD accumulated by glucose-grown cells gradually decreases which is due to the reported previously consecutive induction of the 9α -hydroxylating activity and the accompanying product degrading Δ^1 -steroid dehydrogenating activity in the resting *Rhodococcus* sp. cells [7]. It is known from the literature that bacteria are capable of assimilation of AD through a metabolic pathway proceeding either via 9α -hydroxylation followed by Δ^1 -dehydrogenation or via Δ^1 -dehydrogenation followed by 9α -hydroxylation. In any case, the combined action of the two enzymes leads to formation of the unstable compound 9α -OH-ADD and spontaneous splitting the steroid ring which opens the process of complete AD degradation and its utilization as a single or an additional source of carbon and energy [15]. As the activity of either 9α -steroid hydroxylase or Δ^1 -steroid dehydrogenase prevails in different bacterial strains, this is successfully applied in the manufacturing of two key intermediates for steroid drug synthesis, 9α -OH-AD and ADD, respectively [16].

Figure 2. TEM of glucose-grown *Rhodococcus* sp. cells. Electron dense cell surface (A) and fibrillar structures (B). Bar = 0.5 μm .

Figure 4. Dynamics of 9 α -OH-AD accumulation by glucose- and *n*-alkanes-grown *Rhodococcus* sp. cells.

The process of 9 α -hydroxylation carried out by *n*-alkanes-grown cells, on the contrary to the one carried out by glucose-grown cells, starts almost immediately after adding the AD to the reaction mixture. We find reasonable to explain this lack of *lag*-phase with the enhanced hydrophobicity of the *n*-alkanes-grown cells that in turn provides faster and stronger contact with the crystal steroid substrate. Such explanation is in accordance with the observations showing the growing naturally hydrophobic *Mycobacterium vaccae* [17] and *Arthrobacter simplex* [18] cells attached to the sitosterol particles. In case of the *Mycobacterium fortuitum* cells, they have even been co-grinded with the steroid substrate to reinforce mechanically their contact as to achieve an effective transformation reaction [19].

We are inclined to suggest that in the *n*-alkanes-grown cells the enzyme pathway that allows for complete steroid mineralization may be impaired. Growth on hydrocarbons may lead

Figure 3. TEM of *n*-alkanes-grown *Rhodococcus* sp. cells. Electron-transparent inclusion bodies located in the nucleoid regions (A), closely to the disrupted cell surface membranes (B), and in the area of the mesosome (C). Bar = 0.5 μm .

to possible biochemical and physiological modifications resulting in significant prevalence of the steroid hydroxylation reaction over the reaction of steroid degradation and/or its complete inhibition manifested by the lack of the accompanying product destruction. In this context, possible explanation of our observation may come from the metabolic and physiological responses of *Rhodococcus* cells to the presence of hydrocarbons [20]. Results obtained in our study are also in accordance with the recently published evidences for the positive correlation between the ability of some bacteria for both, assimilation of *n*-alkanes and performing steroid transformation reactions [21, 22].

From this point of view, the ability of *Rhodococcus* sp. for growth on *n*-alkanes and for carrying out an effective 9 α -steroid hydroxylation in growth restricting conditions can be considered as two-parts process. The main peculiarity of this process is that each of its parts is based on a hydrophobic substrate. Thus, the cultivation of the naturally hydrophobic *Rhodococcus* sp. cells on the mixture of *n*-alkanes as a source of carbon and energy in the first part (aiming at harvesting a cell depot with an intentionally increased cell surface hydrophobicity) is of paramount importance. The application of this cell depot in the second part of process, the transformation of crystal AD itself allows for a robust cell-substrate contact and results in a highly effective reaction of 9 α -steroid hydroxylation without product destruction. In this context, the cultivation of the *Rhodococcus* sp. cells on a mixture of *n*-alkanes can be perceived as an adaptation to the presence of the hydrophobic crystalline substrate to be transformed. That is why we believe it is worth to continue studies on the correlation between the ability of *Rhodococcus* to assimilate *n*-alkanes and to transform crystal steroids in growth restricting conditions. Experiments in this area may reveal important knowledge with possible application in engineering of biotechnological steps in the steroid drug manufacturing avoiding both, application of solvents and manipulations usually applied to microbial cells.

4 Concluding remarks

An effective process of 9 α -steroid hydroxylation of androstenedione based solely on the natural characteristics and peculiarities of the *Rhodococcus* sp. cells is presented for the first time. Cells were cultivated on media containing *n*-alkanes as a source of carbon and energy aiming at harvesting a cell depot with an additionally increased cell surface hydrophobicity. Steroid transformation process was carried out by resting *n*-alkanes-grown *Rhodococcus* sp. cells and androstenedione was added in a crystal form. As an outcome, the intentionally growth-driven enhancement of the cell surface hydrophobicity (a consequence of the ability of the naturally hydrophobic *Rhodococcus* sp. cells to assimilate *n*-alkanes) rendered them higher compatibility with the crystal hydrophobic steroid substrate and improved its accessibility in the process of transformation. The ultimate result was an achievement of a significantly increased 9 α -steroid hydroxylating activity and lack of the accompanying product destruction.

Practical application

An effective process of 9 α -steroid hydroxylation of androstenedione was performed by *Rhodococcus* sp. cells with an intentionally increased cell surface hydrophobicity achieved by cultivation of the cells on a medium containing *n*-alkanes as a source of carbon and energy. The transformation process itself was carried out by resting *Rhodococcus* cells in buffer medium where the steroid substrate was added in a crystal form.

The novelty of the suggested approach consists in the employment of the natural ability of bacteria from genus *Rhodococcus* for assimilation of *n*-alkanes and adaptation to their presence via ultrastructural, physiological and metabolic modifications. Thus, via cultivation on a medium containing *n*-alkanes as a sole source of carbon and energy was harvested a cell depot with intentionally increased cell surface hydrophobicity which in turn rendered these cells higher compatibility with the crystal steroid substrate and facilitated its accessibility.

The accomplishment by the *n*-alkanes-grown *Rhodococcus* sp. cells of an almost stoichiometrical transformation of androstenedione into its 9 α -hydroxy-derivative without accompanying product destruction revealed the potential of the suggested approach.

The authors have declared no conflict of interest.

5 References

- [1] Kieslich, K., Biotransformations, in: Rehm, H.J., Reed, G. (Ed.), *Biotechnology. A comprehensive treatise in 8 volumes*, Weinheim: Verlag Chemie 1984, 6A, 1–78.
- [2] Donova, M., Egorova, O., Microbial steroid transformations: current state and prospects. *App. Microbiol. Biotechnol.* 2012, 94, 1423–1447.
- [3] Mutafov, S., Angelova, B., Dacheva, V., Influence of methanol, dimethyl sulfoxide and dimethylformamide on the 9 α -hydroxylation activity of *Rhodococcus* sp. cells. *F.E.C.S. Fifth international conference on Chemistry and Biotechnology of biologically active natural Products*, 1989, Varna, Bulgaria, Conference Proceedings, 4, 396–400.
- [4] Angelova, B., Mutafov, S., Avramova, T., Dimova, I. et al., 9 α -Hydroxylation of 4-androstene-3,17-dione by resting *Rhodococcus* sp. cells. *Proc. Biochem.* 1996, 31, 179–184.
- [5] Avramova, T., Spassova, D., Mutafov, S., Momchilova, S. et al., Effect of Tween 80 on 9 α -steroid hydroxylating activity and ultrastructural characteristics of *Rhodococcus* sp. cells. *World J. Microbiol. Biotechnol.* 2010, 26, 1009–1014.
- [6] Angelova, B., Fernandes, P., Cruz, A., Pinheiro, H. M. et al., Hydroxylation of androstenedione by resting *Rhodococcus* sp. cells in organic media. *Enz. Microb. Technol.* 2005, 37, 718–722.

- [7] Mutafov, S., Angelova, B., Avramova, T., Boyadjieva, L. et al., The inducibility of 9α -steroid hydroxylating activity in resting *Rhodococcus* sp. cells. *Proc. Biochem.* 1997, 32, 585–589.
- [8] Rosenberg, M., Gutnik, D., Rosenberg, E., Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* 1980, 9, 29–30.
- [9] Christie, W., *Lipid Analysis*. The Oily Press, Bridgwater, England, 2003, p. 208.
- [10] Sorkhoh, N., Ghannoum, M., Ibrahim, A., Stretton, R. J., Sterols and diacylglycerophosphocholines in the lipids of the hydrocarbon-utilizing prokaryote *Rhodococcus rhodochrous*. *J. Appl. Bacteriol.* 1990, 69, 856–863.
- [11] Hamilton, J., McRoberts, W., Larkin, M., Harper, D., Long-chain haloalkanes are incorporated into fatty acids by *Rhodococcus rhodochrous* NCIMB 13064. *Microbiology* 1995, 141, 2611–2617.
- [12] Kölbl, H., Electron microscopy, in: Kubica G., Wayne, L. (Ed.) *The Mycobacteria. A source book (in two parts). Part A*, Marcel Dekker, Inc., New York and Basel, 1984, p. 253.
- [13] Alvarez, H., Mayer, F., Fabritius, D., Steinbüchel, A., Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. *Arch. Microbiol.* 1996, 165, 377–386.
- [14] Kim, I., Foght, J., Gray, M., Selective transport and accumulation of alkanes by *Rhodococcus erythropolis* S+14He. *Biotechnol. Bioeng.* 2002, 80, 650–659.
- [15] Miclo, A., Germain, P., Lefebvre, G., Catabolism of androst-4-ene-3,17-dione by mutant strains of *Nocardia restricta*. *J. Basic Microbiol.* 1986, 26, 83–89.
- [16] Fernandes, P., Cruz, A., Angelova, B., Pinheiro, H. et al., Microbial conversion of steroid compounds: recent developments. *Enz. Microb. Technol.* 2003, 32, 688–705.
- [17] Hörhold, C., Gottschaldt, B., Grosse, H.-H., Microbial transformation of sterols to androstane-compounds in presence of organic resins. *F.E.C.S. Fifth international conference on Chemistry and Biotechnology of biologically active natural Products*, 1989, Varna, Bulgaria, Conference Proceedings, 4, 92–110.
- [18] Rajkhowa, R., Goswami, P., Singh, H., Mode of uptake of sterol by *Arthrobacter simplex*. *World J. Microbiol. Biotechnol.* 2000, 16, 63–68.
- [19] Atrat, P., Hösel, P., Richter, W., Meyer, H. et al., Interactions of *Mycobacterium fortuitum* with solid sterol substrate particles. *J. Basic Microbiol.* 1991, 31, 413–422.
- [20] Laczi, K., Kis, Á., Horváth, B., Maróti, G. et al., Metabolic responses of *Rhodococcus erythropolis* PR4 grown on diesel oil and various hydrocarbons. *Appl. Microbiol. Biotechnol.* 2015, 99, 9745–9759.
- [21] Liu, X., Wang, Z.-B., Wang, Y.-N., Kong, J.-Q., Probing steroidal substrate specificity of Cytochrome P450 BM3 variants. *Molecules* 2016, 21, 760.
- [22] Liu, X., Kong, J.-Q., Steroids hydroxylation catalyzed by the monooxygenase mutant 139-3 from *Bacillus megaterium* BM3. *Acta Pharmacol. Sin.* 2017, 7, 510–516.