# OPTIMIZATION OF GROWTH CONDITIONS FOR THE TESTOSTERONE-PRODUCING *MYCOLICIBACTERIUM NEOAURUM* STRAIN

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**Abstract.** Microbial production of testosterone from phytosterol is a promising alternative to the chemical synthesis from androstenedione, which is currently used. It is possible using wild-type or recombinant strains of *Mycolicibacterium neoaurum*. One of the key steps of biotechnology development is inoculum preparation which determines the volumetric/mass ratios of the bioreactors and affects the biotransformation rate and duration. In the present work, the conditions for the growth of testosterone-producing strain of *Mycolicibacterium neoaurum* recombinant strain with enhanced 17β-HSD activity were optimized. The effects of medium composition, temperature, and glucose supplements on biomass size and density were estimated. The results are of importance for the development of microbial technology for testosterone production.

**Keywords:** testosterone, biotechnology, mycolicibacteria, recombinant strain.

**List of Abbreviations**

AD – androst-4-ene-3,20-dione

ADD – androsta-1,4-diene-3,20-dione

17β-HSD – 17β-hydroxysteroid dehydrogenase

1816Rec – *M. neoaurum* VKM Ac-1816D recombinant strain

1815Rec – *M. neoaurum* VKM Ac-1815D recombinant strain

## Introduction

Testosterone (androst-4-en-3-one-17β-ol) is an androgen steroid which plays an important role in the regulation of vital processes and is widely used in medicine for hormone replacement therapy and the treatment of endocrinological disorders. The complete chemical synthesis of testosterone is difficult due to the asymmetric structure of the steroid nucleus. Currently, testosterone is produced from androst-4-en-3,20-dione (AD) using chemical or enzymatic methods (Perez *et al*., 2006). However, medical application of the chemically synthesized testosterone may cause undesirable side effects (Sood *et al*., 2017).

Microbial testosterone production is a promising alternative to its chemical or chemoenzymatic synthesis. The most attractive way is its production from cheap and available phytosterols that is possible using sterol-transforming actinobacteria such as *Mycolicibacterium neoaurum*. These bacteria are capable of the degrading phytosterol side chain to form AD and possess 17β-hydroxysteroid dehydrogenase (17-HSD) activity that results in testosterone accumulation (Lo *et al*., 2002; Egorova *et al*., 2009). Recently, effective testosterone production has been reported based on the successive application of *M. neoaurum* and *Nocardioides simplex* strains in one bioreactor (Tekucheva *et al*., 2022).

Recombinant strain producing testosterone from phytosterol has been created by expression of fungal 17β-HSD in *Mycolicibacterium smegmatis* $mc^2$ 155 (Fernández-Cabezón *et al*., 2017). In our laboratory, the recombinant strains of AD-producing *M. neoaurum* VKM Ac-1815D (formerly, *Mycobacterium* sp. VKM Ac-1815D) and ADD-producing *M. neoaurum* VKM Ac-1816D (formerly, *Mycobacterium* sp. VKM Ac-1816D) have been constructed that demonstrated efficient testosterone production (Strizhov *et al*., 2016; Karpov *et al*., 2016).

When developing biotechnology, an important step is to obtain an inoculum that is further used in batch fermentation. The quality,

growth status and density of the inoculum greatly affect phytosterol bioconversion rate and duration; determine a volumetric/mass ratio of bioreactors at the scaling up of biotechnology. Noteworthy, the recombinant strains may differ from their parent organisms in the growth characteristics.

In this work, the effect of the medium composition, temperature and glucose supplement on the growth of the wild-type and recombinant strains of *M. neoaurum* strains was studied, and the regimen for obtaining of dense culture of recombinant *M. neoaurum* VKM Ac-1816D was optimized.

## Materials and Methods

*Reagents*

The following reagents were used: yeast extracts (No. 1, Pronadisa, Spain; No. 2, Helicon, France; No. 3, Difco, United States; No. 4, Biospringer, France); kanamycin (Biokhim, Russia). Other reagents of chemical or analytical purity were purchased from domestic suppliers.

*Microbial strains and cultivation*

The strains *M. neoaurum* VKM Ac-1815D (formerly, *Mycobacterium* sp. VKM Ac-1815D) and *M. neoaurum* VKM Ac-1816D (formerly, *Mycobacterium* sp. VKM Ac-1816D) were obtained from the All-Russian Collection of Microorganisms (VKM), G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences (IBPM RAS). The recombinant *M. neoaurum* VKM Ac-1815D (hereinafter, 1815Rec) and *M. neoaurum* VKM Ac-1816D (hereinafter, 1816Rec) strains were obtained from the Laboratory of Microbial Transformation of Organic Compounds, IBPM RAS.

The inoculum was grown in two generation stages. The first inoculum was grown in 100 ml medium composed of (g/l): $K_2HPO_4 \times 3H_2O$ – 0.5; $KH_2PO_4$ – 0.5; $(NH_4)_2HPO_4$ – 1.5; $MgSO_4 \times 7H_2O$ – 0.2; $FeSO_4 \times 7H_2O$ – 0.005; $ZnSO_4 \times 7H_2O$ – 0.002; glucose – 12.5; yeast extract – 10, pH 7.0. The seed material taken from the solid medium was 250±7 mg/l (d.w.). The recombinant strains were cultured on the same medium supplemented with kanamycin (20 μg/ml). Cultivation was carried out for 48 hours at 30 °C, aerobically (200 rpm), in 750 ml shaking flasks containing 100 ml medium. Then, 10% (v/v) of the first generation seed culture was used to inoculate 100 ml of the same medium, and cultivated for 24 hours under the same conditions.

To analyze the physiological characteristics of wild type and recombinant strains of mycolicibacteria, 95 ml of the medium was seeded with the 5% second generation inoculum and cultivated for 72 hours as described above. Initial glucose concentration varied from 15 to 40 g/l, yeast extract – from 12 to 32 g/l, and the seed dose was increased by 2- and 4-fold.

The wild-type *M. neoaurum* VKM Ac-1815D and *M. neoaurum* VKM Ac-1816D (1815D and 1816D, respectively) and recombinants (1815Rec and 1816Rec) strains growth was estimated by biomass (dry weight, d.w.), optical density ($OD_{600}$), and colony-forming units counting (CFU) methods. For this, the 5 ml of liquid culture was taken at the 0th, 12th, 24th, 36th, 48th and 72th hours of cultivation. For dry weight estimation, the 2 ml aliquot was centrifuged at 13,200 x *g*, for 10 min, and dried at 110°C for 24 hours; then the bacterial biomass was weighed. $OD_{600}$ of the each taken aliquot was measured using a spectrophotometer (Eppendorf, US) at 600 nm according to manufacturer's recommendations. For CFU counting, some samples were serially diluted with saline under vigorous agitation and plated on the solid medium of the above mentioned composition supplied with agar (20 g/l).

Glucose assay was carried out in accordance with (Gusakov *et al*., 2011); optical density was measured using a 2-beam spectrophotometer (Shimadzu, Japan) at 600 nm. Glucose concentration was calculated using calibration curve.

The recombinant strain 1816Rec growth was assayed at different temperatures (30–36°C) in order to determine the optimum temperature for its cultivation.

*Statistical data processing*

The experiments were carried out in no less than three replicates. All measurements were carried out in at least 3 repetitions; for each

sample, the standard deviation and confidence interval for the normal distribution were calculated at a significance level of 0.95.

**Results**

The growth of the strains was estimated by $OD_{600}$, dry weight and CFU methods. As follows from Fig. 1, the parental strains (*M. neoaurum* VKM Ac-1815D and Ac-1816D) slightly preferred their recombinant derivatives in the growth rate. The differences in the growth dynamics (Fig. 1A *vs*. Fig. 1B) were mainly due to the clumping of the cells after 48 hours that was higher in the case of 1816D. The strain of 1815DRec demonstrated slower growth compared to the respective parent strain, while the difference in the growth rates between the parent and recombinant strains of 1816D was insignificant (Fig. 1B, 1C).

Initial rates of glucose consumption were generally comparable for all the strains being slightly higher for the recombinants. The rate of glucose utilization increased after 36 hours in all the cases (Fig. 2A).

As reported earlier, higher yield of testosterone was achieved when using the recombinant strain 1816Rec for phytosterol bioconversion (Karpov *et al*., 2016). Further experiments were carried out using this strain (Fig. 2B).

The seed dose in a range of 250 – 1000 mg/l (d.w.) had no remarkable effect on the growth of the strain (Fig. 3). It reached 8±0.06 g/l (d.w.), $OD_{600}$ 19±0.04 and CFU $15 \cdot 10^{10} \pm \pm 0.9 \cdot 10^{10}$ cells/ml for 48 hours. The doubling time of the recombinant strain was estimated as 5.9±0.7 hours.

The data obtained allow us to conclude that the duration of the cultivation process should not exceed 48 hours and 48 hours for the first and second generation culture, respectively. In addition, the results achieved indicate that it is possible to use of a 5% seed dose for the second generation culture.

The growth of the strain depended on glucose content in the medium (Fig. 4). The data obtained using $OD_{600}$, biomass and CFU estimation differed, but allowed to conclude that 20 g/l glucose is optimal for the strain growth.

Yeast extract is commonly used as a component in the complex media for biotechnological processes. However, its composition often varies depending on the supplier. In this work, we tested the effect of yeast extracts from different suppliers on growth characteristics of the 1816Rec strain (Fig. 5). The strain growth depended on the yeast extract applied, and the samples No. 1 and No. 3 provided higher growth outputs. In further experiments yeast extract No. 3 (16 g/l) was applied.

The effect of temperature on the growth of the recombinant strain was estimated in a range of 30–36 °C (Fig. 6A). The culture reached maximum of $OD_{600}$ at 30–32 °C, while low $OD_{600}$ was observed at higher temperatures (Fig. 6A).

Surprisingly, the difference in glucose consumption rates was not so remarkable. It was some higher at the initial stage (for around 6 hours) at 34 °C, but further slowed down for the temperatures of 34-36 °C, and increased for 30-32 °C.

It is known that the temperature determines the rate of all metabolic processes. Thus, after 6 hours of cultivation, at the temperature over 32 °C foaming began with the removal of 1816Rec cells into stable foam. By 30 hours of incubation at the temperature of 36 °C, the optical density of 1816Rec dropped significantly, and the growth stopped. Data on glucose uptake as a function of temperature are consistent with the results of the study of 1816Rec growth ($OD_{600}$) with temperature increasing from 30 °C to 36 °C. As shown in Figure 6A, a temperature in the range of 30-32°C is optimal for the growth of 1816Rec. All subsequent experiments were carried out at 30°C.

As a result of this study, a scheme was proposed for obtaining 1816Rec dense culture through two generations on a medium with glucose (20 g/l) and yeast extract (16 g/l), which makes it possible to inoculate a productive medium with phytosterol by 5% seed dose and achieve satisfactory rates in terms of testosterone yield.

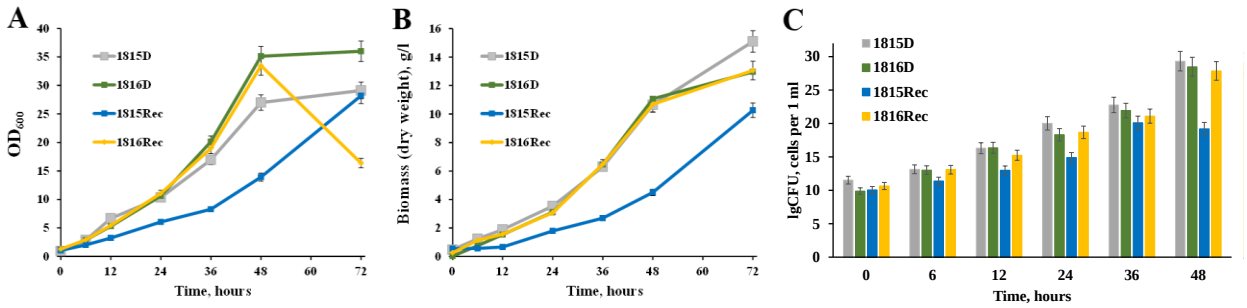


**Fig. 1.** Growth of *M. neoaurum* VKM Ac-1815D and *M. neoaurum* VKM Ac-1816D wild-type (1815D and 1816D) and recombinant (1815Rec and 1816Rec) strains, respectively. A – $OD_{600}$; B – Biomass; C – lg CFU

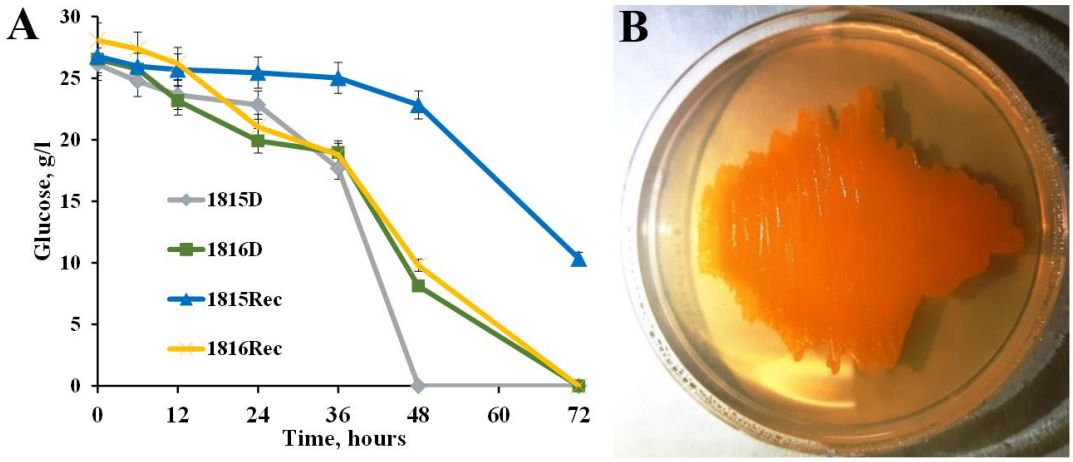


**Fig. 2.** Glucose consumption by parental (1815D and 1816D) and recombinant (1815Rec and 1816Rec) mycolicibacterial strains (A). Recombinant strain 1816Rec on a Petri dish (B)

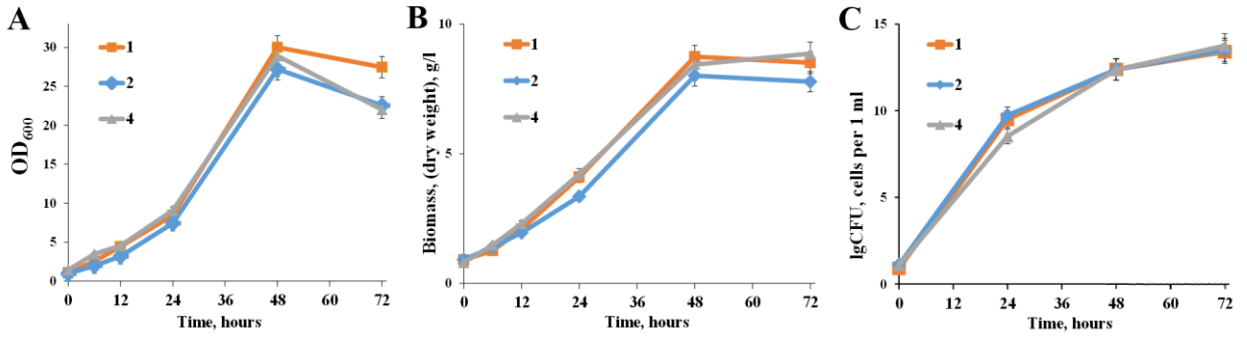


**Fig. 3.** Growth of the recombinant mycolicibacterial strain 1816Rec depending on the seed dose. A – $OD_{600}$; B – Biomass; C – lg CFU. 1 – Control, seed dose is 250±7 mg/l (d.w.); 2 – 2-fold seed dose, 500±24 mg/l (d.w.); 4 – 4-fold seed dose, 1000±38 mg/l (d.w.)

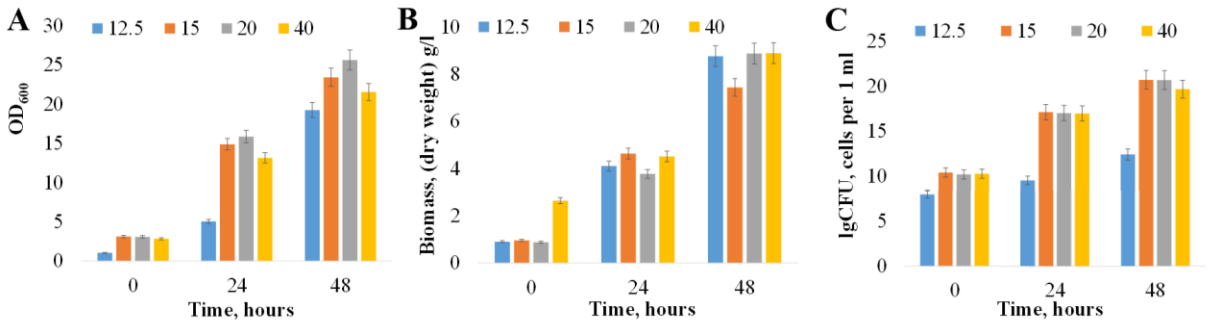


**Fig. 4.** Glucose concentration effect on growth characteristics of Ac-1816Rec. A – $OD_{600}$; B – Biomass; C – lgCFU

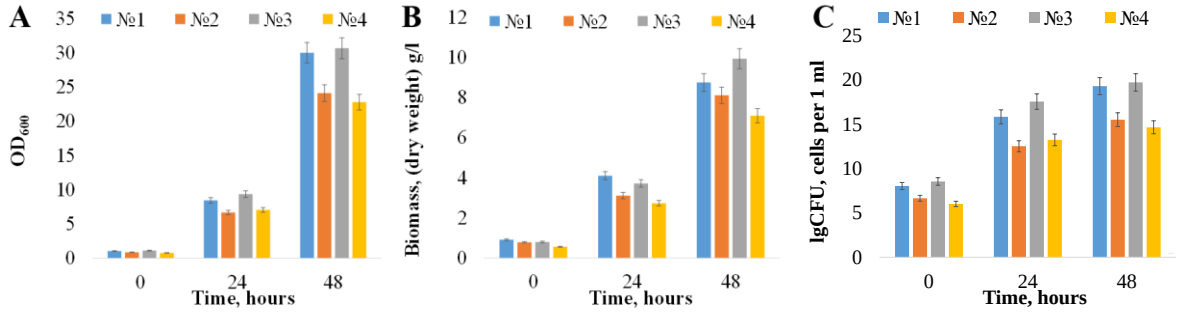


**Fig. 5.** Effect of yeast extracts on the growth of the recombinant mycolicibacterial strain 1816Rec. A – $OD_{600}$; B – Biomass; C – lgCFU

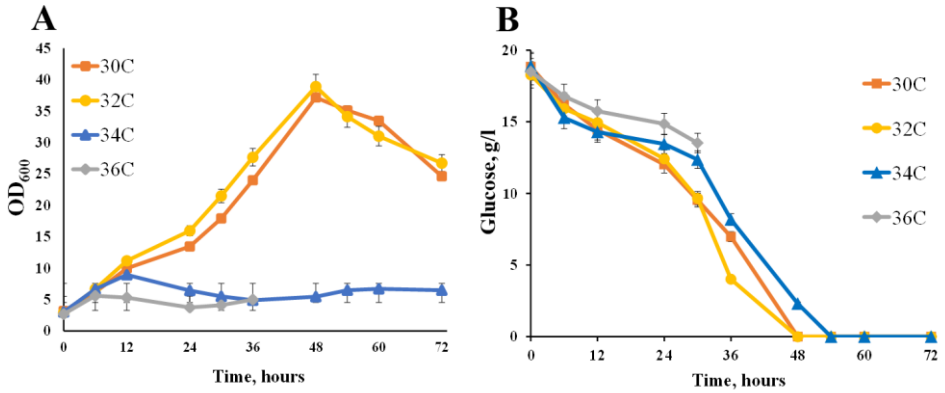


**Fig. 6.** Effect of temperature on the recombinant strain 1816Rec growth ($OD_{600}$) (A) and glucose utilization (B)

## Discussion

An actual problem of modern biotechnology is the production of steroid medical compounds, including testosterone. The strains 1815D and 1816D as well as their recombinants are capable of producing testosterone from phy-tosterol. One of the points of this work was to study growth characteristics of recombinant mycolicibacteria in comparison with the wild-type 1815D and 1816D strains, which produce AD and ADD from phytosterol, respectively. As it turned out, the recombinant strain 1816Rec has growth characteristics similar to those of the parent strain 1816D including the same doubling time and maximum biomass yields.

The optimization of the growth conditions for the recombinant strain 1816Rec was carried out, including the study of the influence of the seed dose, carbon and organic nitrogen sources, as well as the influence of temperature on the growth and glucose consumption of this strain. When studying the effect of glucose, it was shown that at its concentration of 20 g/l, the growth indicators of the inoculum reached the maximum values, a twofold increase in the glucose load led to inhibition of the growth of recombinant mycolicibacteria.

Yeast extract due to its low cost and rich content of various amino acids, peptides, water-soluble vitamins, growth factors, trace elements and carbohydrates is commonly used as the main component of the culture medium (Zhang *et al*., 2003). The composition of yeast extract varies due to complex substrates and poorly controlled fermentation conditions by yeast producers. This composition variability often leads to inconsistent fermentation characteristics. Thus, batches from the same yeast extract manufacturing process were reported to influence the bacterial biomass levels and growth rates about up to 50% (Zhang *et al*., 2003). In our experiments the difference between the growth outputs on yeast extracts of different brands was from 5% to 29%, and the yeast extract providing higher 1816Rec growth was chosen.

Temperature is known to play a crucial role in regulating the activity and growth of microorganisms (Madigan *et al*., 2003). The temperature influence on cellular processes in cultured bacteria is well understood, and it is also known that the metabolic rate may double for every 10°C increase in temperature (Morita 1974). Also, the temperature dependence of various biochemical processes can vary greatly. The disparate effects of temperature have been described, for example, on the uptake of various forms of inorganic nitrogen and various amino acids, enzymatic activity, and variability in the relationship of cellular respiration to ATP production. Moreover, temperature change experiments performed on bacterial cultures have revealed differences in the response of cell growth to respiration, indicating that temperature-dependent differences appear at many levels of cellular organization (Apple *et al*., 2006). Our study on the temperature effect on glucose consumption rate and growth of 1816Rec showed that a temperature in the range of 30-32°C is optimal for this strain.

## Conclusion

Optimization of the growth conditions for the recombinant micolicibacterial strain 1816D with enhanced 17β-HSD activity which performs phytosterol conversion to testosterone was conducted. The effect of seed dose, glucose concentration, some yeast extracts from different suppliers and cultivation conditions on the culture growth characteristics was revealed. A scheme for obtaining two generations seed culture in a growth medium of an optimized composition that makes it possible to achieve high rates of target activity towards producing testosterone by the recombinant strain 1816Rec was proposed.

## Acknowledgements

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