

Effects of Different Carbon Sources on Growth, Membrane Permeability, β -Sitosterol Consumption, Androstadienedione and Androstenedione Production by *Mycobacterium neoaurum*

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Abstract Effects of different carbon sources on growth, membrane permeability, β -sitosterol consumption, androstadienedione and androstenedione (AD(D)) production by *Mycobacterium neoaurum* were investigated. The results indicated that glucose was advantageous to the growth and resulted in the adverse effects on the phytosterols consumption and AD(D) production compared to the results of propanol and isopropanol as sole carbon source. The cell wall widths of 9.76 by propanol and 8.00 nm by isopropanol were 38.3 and 49.4 % thinner than that of 15.82 nm by glucose, respectively. The partition coefficient of the cell grown in propanol and isopropanol was 18.1 and 22.2, which were 7.23- and 9.09-fold higher than that of the cell grown in glucose.

Keywords Carbon sources · Membrane permeability · 1,4-Androstadiene-3,17-dione · 4-Androstene-3,17-dione · *Mycobacterium neoaurum*

1 Introduction

1,4-Androstadiene-3,17-dione (androstadienedione, ADD) and 4-androstene-3,17-dione (androstenedione, AD) are the major compounds in the synthesis of certain steroid drugs. Many of these steroids are essential substances [1]. ADD and AD (AD(D)) are intermediates in the synthesis of several corticoids (cortisol, prednisolone, etc.), androgens

(testosterone and its derivatives), gestagens (acetomepregenol, 17α -hydroxyprogesterone esters, etc.), diuretics (canrenone and spironolactone) and anabolic compounds (boldenone and methandrostenolone) [2]. The size of the world market for AD(D) compounds exceeds, 1000 tons per year [3]. Biotransformation with microorganisms such as *Mycobacterium neoaurum* was regarded the most promising strain to transform phytosterols (including β -sitosterol, campesterol, brassicasterol and stigmasterol) into AD(D) that performed side-chain cleavage of the substrate molecules [2, 4].

However, the reaction of biotransformation encountered some difficulties for the *Mycobacterium* genus itself inhibited the substrate (e.g., β -sitosterol, sitosterol, and stigmasterol) into the cell and may be partially responsible for the slow growth rate of mycobacteria by restricting uptake of nutrients into the organisms [5] and both the substrate and AD(D) have low solubilities in the aqueous medium [6]. Cell wall and cell membrane act as barriers to the transport of substrates into the cytoplasm and thus severely affects the overall yield of the biotransformation product. Borrego et al. [7] reported that the nature of carbon source affects the cell wall composition of mycobacterial cells. Dias et al. [8] also reported an increase in yield of AD from tall-oil biotransformation when glycerol was used as carbon source instead of fructose.

Since many biocatalysis substrates are hydrophobic and/or relatively big molecules, obtaining rapid substrate permeation through outer membranes is a common and formidable obstacle in whole-cell biocatalysis. The outer membrane of Gram-negative bacteria [9] excludes even small hydrophobic compounds. The rate of entry of both hydrophobic and hydrophilic molecules is often the rate-limiting step of a biocatalysis reaction. We found different carbon sources such as glucose, propanol and isopropanol

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remarkably affected biotransformation process by *M. neoaurum* in our former study [4]; therefore, this paper described the effects of carbon sources on the membrane permeability, growth, β -sitosterol consumption, AD(D) production by *M. neoaurum*.

2 Materials and Methods

2.1 Maintenance and Preculture of Bacteria

Mycobacterium neoaurum, described by Wang and Yu [4], was maintained on potato dextrose agar slants at room temperature. A preculture of *M. neoaurum* was grown in medium composed of the following (g/l): glucose 20 g, yeast extract 5 g, MgSO_4 2 g, potassium dihydrogen phosphate 3 g and disodium hydrogen phosphate 1 g in 1 l tap water (pH 7.0).

2.2 Chemicals

β -Sitosterol, containing 16.52 % campesterol, served as a transformation substrate (purchased from Hubei Hengshuo Chemical Co., Ltd, Hubei Province, PR. China). 4-Androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) standards were obtained from SIGMA. All other chemicals used were of reagent grade.

2.3 Biotransformation Trials

After 2 days of cultivation, 3 ml of preculture was added to the biotransformation medium (30 ml in a 250 ml flask) which contained the following (g/l): carbon source 20 (glucose, propanol or isopropanol as sole carbon source, respectively), yeast extract 2, ammonium nitrate 2, magnesium sulfate anhydrous 2, potassium dihydrogen phosphate 3 and disodium hydrogen phosphate 1 in 1 l tap water (pH 7.0). To investigate the effects of different carbon sources on growth, membrane permeability, β -sitosterol consumption, androstadienedione and androstenedione production after inoculation with the growth medium, 10 g/l β -sitosterol dissolved in 0.8 % Tween 80 was added into the broth for biotransformation. The biotransformation was carried out at 28 °C and 220 rpm. Each experiment was performed twice at the same time to check the reproducibility.

2.4 Analytical Methods

At every 8 h of the incubation period, 5 ml of culture broth was taken from the flask and centrifuged. The centrifuged

cells (5000g, 10 min) were washed twice in distilled water, and the biomass was dried at 80 °C for 24 h and was measured as cell dry weight (CDW); 3 ml of supernatants was extracted with 6 ml ethyl acetate by vigorously mixing for 10 min in a separatory funnel. The organic layer was evaporated and dried. The residue was then dissolved in 1.0 ml mobile phase. A 100 μl of sample diluted with mobile phase was filtered through a 0.45- μm nylon syringe filter and transferred to a sampling vial. Samples (20 μl) were analyzed by HPLC with equipment consisting of a solvent delivery system (DIONEX 3300, USA) and a variable wavelength UV–Vis detector (L 7420, Hitachi, Japan) set to 244 nm, equipped with a sample injector (7725i, Rheodyne, USA) fitted with a 20- μl sample loop. We used an Eclipse XDB-C18 column (250 mm \times 4.6 mm i.d.; 5- μm particle diameter, 250 Å average pore size) (Agilent, USA) with a guard column holder using distilled water/methanol (35/65, v/v) as the mobile phase at a flow rate of 1.0 ml min^{-1} . The retention times of the biotransformation products were compared to those of AD and ADD, at 11.465 and 7.811 min, respectively. Calculations of the obtained weight of AD(D) were determined by the calibration curve of authentic reference samples using the area under the curve of biotransformation products in each chromatogram.

For transmission electron microscopy (TEM), the cells grown in the synthetic medium with the different carbon sources for 144 h at 28 °C and 220 rpm were harvested at 5000 g for 10 min and washed twice with PBS. The cells were suspended in Cadillac fixative (2.5 % glutaraldehyde, 1.2 % acrolein in sym-collidine) at room temperature followed by washing with 0.1 M cacodylate buffer twice for 5 min each. The cells were subjected to postfixation in 1 % osmium tetroxide for 90 min at room temperature followed by resuspending the pellet once in 0.1 M cacodylate buffer for 10 min, washed with distilled water for 10 min and dehydrated with ascending grades (30, 50, 70, 95, 100 and 100 % again) of ethanol. The pellet was then washed with a solution of ethanol and propylene oxide at 1:1 ratio for 10 min followed by propylene oxide for 10 min twice. Infiltration was done in Spurr's resin containing propylene oxide at 1:1 ratio for 5 h under vacuum and at 3:1 ratio for 10 min twice under vacuum. Embedding was done in Spurr's resin under vacuum at 60 °C for 48 h [10]. Blocks of 70–80 nm thickness were cut in Ultratome III (LKB) ultramicrotome and were stained according to [11]. Thin sections were examined in a JEM 100 S (Jeol) electron microscope at 80 kV. The figures obtained by electron microscopy were used to determine the differences between the cell wall ultrastructure of the melanotic and albino strains. Twenty cells for each strain were

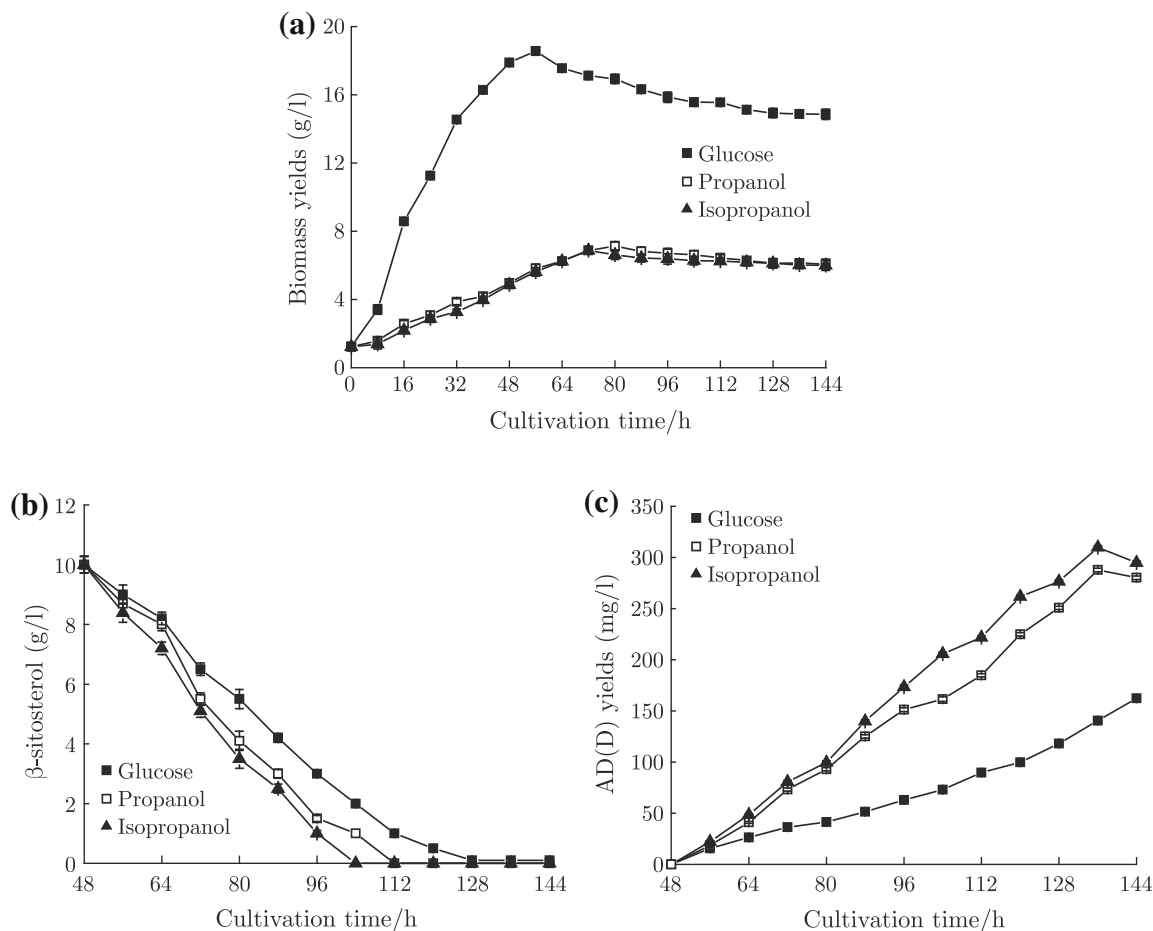


Fig. 1 Effect of different carbon sources on growth, β -sitosterol consumption and AD(D) production by *M. neoaurum*

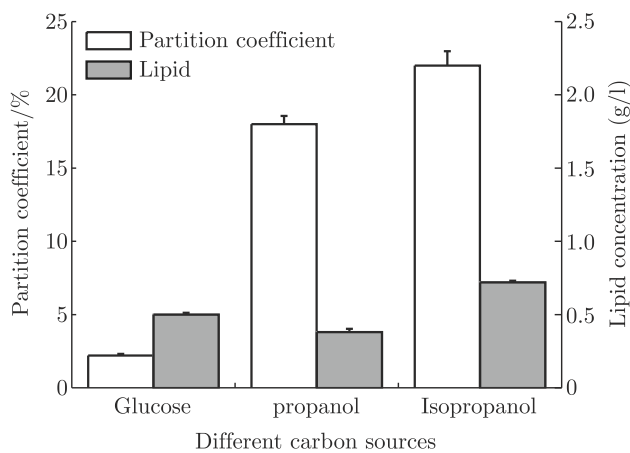


Fig. 2 Effects of different carbon sources on the cellular hydrophobicity (partition coefficient, PC) and lipids amount of *M. neoaurum*

measured using Adobe Photoshop 7.0.1. The diameter of the cell and the thickness of the cell wall were determined, and the mean and standard deviation were

calculated using Microsoft excel. The ratio of cell size (without cell wall size) to cell wall size was determined.

For cellular hydrophobicity determination, an equal volume of hexane (1:1) was added to the cultures grown for 144 h with glucose, glycerol or isopropanol, respectively. The mixtures were shaken well. The solvent with the cells was harvested, and both aqueous and solvent phases were evaporated in vacuum and the weight was measured.

The whole-cell fatty acids were extracted from the freeze-dried biomass according to the method of [12]. Gas chromatography mass spectrometry (GC-MS) analyses were carried out using Agilent 6890N equipment (USA). A capillary column (HP-5, 5 % phenyl siloxane, USA) of 30 m \times 0.32 mm was used in the gas chromatograph. The carrier gas, helium, was used at an inlet pressure of 0.8 ml min⁻¹. The column temperature was programmed from 80 to 280 °C, to 8 °C min⁻¹; both the injector and the interphase temperatures were kept at 280 °C. The ionization was performed at 70 eV, and the ion source temperature was 250 °C.

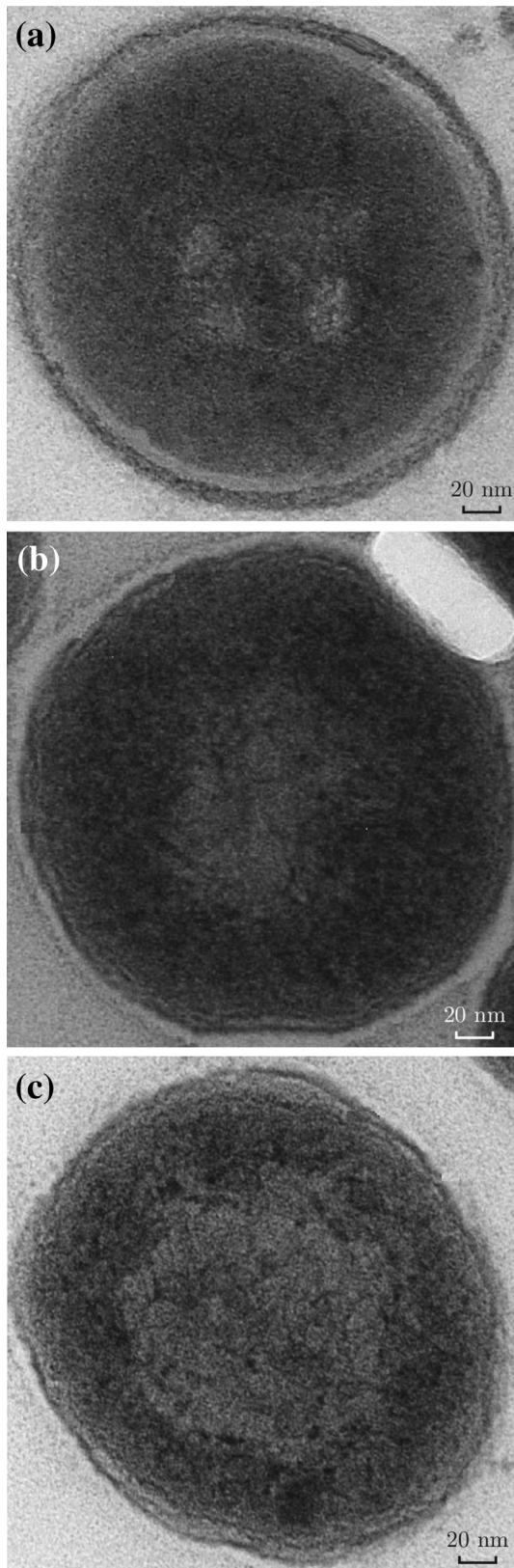


Fig. 3 Effects of different carbon sources on width of cell wall of *M. neoaurum* by transmission electron microscopy. *M. neoaurum* grown in synthetic medium with: **a** glucose (cell wall of 15.82 nm); **b** propanol (cell wall of 9.76 nm); and **c** isopropanol (cell wall of 8.00 nm) during 144 h at 30 °C, 220 rpm. The arrows show the cell wall. Bar 20 nm

3 Results and Discussion

3.1 Effects of Carbon Sources on Growth, β -Sitosterol Consumption, and AD(D) Production by *M. neoaurum*

Effects of carbon sources on growth, β -sitosterol consumption, and AD(D) production by *M. neoaurum* were studied. Figure 1a shows the cell growth kinetics in the presence of different carbon sources, the lag phase was shortened substantially from 16 to 4 h, and the cell quickly reached the maximal biomass after cultivation for 56 h when employing glucose as carbon source, whereas it was 80 h compared to the results of propanol or isopropanol. Carbon source affected the biomass level, and its peak value of 18.57 g/l was obtained by glucose at 56 h, while the optimal biomass was 7.12 and 6.88 g/l at 80 h when using propanol or isopropanol as carbon sources, respectively. The results indicated that carbon sources had significant effects on the cell growth during fermentation and glucose seemed to be much better for cell growth by *M. neoaurum*. Time courses of residual β -sitosterol concentration were compared in Fig. 1b. When employing isopropanol as carbon source the substrate consumption rate was higher than in other cases. As depicted in Fig. 1c, the results of isopropanol revealed a clear positive influence on the AD(D) yields, and the maximum AD(D) concentration of 312.6 mg/l was obtained at 136 h, while glucose led to the optimal AD(D) concentration of 167.3 mg/l at 144 h. It indicated that glucose was advantageous to the growth by *M. neoaurum* but not increased the AD(D) production, while isopropanol inhibited the growth but was propitious to the AD(D) accumulation by *M. neoaurum*.

3.2 Effects of Carbon Sources on the Membrane Permeability by *M. neoaurum*

Cell wall and cell membrane are the main barriers to the transport of substrates into the cytoplasm; therefore, we investigated some parameters about the cell wall and membrane permeability such as width of cell wall, lipid content and cellular hydrophobicity, which are correlative to the membrane permeability.

Table 1 Significance analysis of Fig. 2

	<i>P</i> value (glucose vs. propanol)	<i>P</i> value (propanol vs. isopropanol)	<i>P</i> value (isopropanol vs. glucose)
Partition coefficient (%)	***	*	***
Lipid concentration (g/l)	–	**	*

Student's *t* test; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001

Table 2 Effects of different carbon sources on the main fatty acids content by *M. neoaurum*

Fatty acids	Glucose (%)	Propanol (%)	Isopropanol (%)
Oleic acid	26.82 ± 0.68	34.49 ± 1.21	32.21 ± 1.15
Margaric acid	12.88 ± 0.13	0.31 ± 0.03	0.42 ± 0.05
Myristic acid	5.91 ± 0.12	2.92 ± 0.12	2.64 ± 0.09
Stearic acid	4.62 ± 0.11	7.35 ± 0.69	8.12 ± 0.19
Palmitoleic acid isomer	4.24 ± 0.10	–	–
Arachidic acid	3.18 ± 0.11	–	–
Palmitic acid	0.70 ± 0.07	17.60 ± 0.51	17.80 ± 0.48
Tuberculostearic acid	0.40 ± 0.05	7.60 ± 0.22	10.50 ± 0.33
Behenic acid	0.30 ± 0.04	3.8 ± 0.15	5.60 ± 0.19
Myristoleic acid isomer	0.30 ± 0.03	–	–

–, Indicates the absence of a compound

3.3 Effects of Different Carbon Sources on the Cellular Hydrophobicity and Lipids Amount by *M. neoaurum*

As shown in Fig. 2, the results showed that different carbon sources influenced the partition coefficient, lipid content. The hydrophobicity level was highest in isopropanol, followed by propanol and was much higher compared to that of glucose. The partition coefficient of the cell grown in propanol and isopropanol was 18.1 and 22.2, which were 7.23- and 9.09-fold higher than that of the cell grown in glucose. Ratledge [13] reported that in mycobacteria the total lipids accumulated could be affected by the carbon source metabolized. For this reason, the hydrophobicity tendency, the concentration of the total lipids and the composition of the fatty acids were analyzed. The level of lipid in the cells grown in isopropanol was the highest (Fig. 2); besides, in propanol, in contrast to the cellular hydrophobicity, the level of lipid was the lowest. This result suggests that the cellular hydrophobicity could depend not only on the amount of lipid, but also on fatty acid's composition.

3.4 Effects of Carbon Sources on Cell Wall Width by *M. neoaurum*

The width of cell wall by *M. neoaurum* was determined employing TEM, and the results are shown in Fig. 3. The cells wall is 15.82 nm wide when the microorganism was

grown in glucose, while cells grown in propanol and isopropanol had cell wall widths of 9.76 and 8.00 nm, respectively. These results suggested that there was not always a correspondence between width of cell wall and the cell wall composition, since the organism grown in isopropanol had a very thin cell wall, but the cells are very hydrophobic. These results suggest that the carbon source in culture media plays an important role in cellular hydrophobicity.

3.5 Effects of Carbon Sources on the Fatty Acids Content by *M. neoaurum*

The variability of the individual fatty acid isolated from the cells is shown in Table 1. A remarkable difference in the fatty acid was observed in cells grown in glucose, propanol and isopropanol. Arachidic acid and palmitoleic acid isomer were detected when the strain was grown on glucose but not checked in the propanol and isopropanol cases, whereas oleic, palmitic and stearic acids were the principal fatty acids isolated from the cells grown in propanol and isopropanol. Hence, the cellular hydrophobicity is presumably dependent on the fatty acid's composition (Table 2).

Whole cell catalyzed reactions have been found to be 10–100-fold slower than the reactions catalyzed by free enzymes [14]. Weakening the cell permeability barriers without affecting viability has been employed as a procedure for improving sterol biotransformation efficiency [14–

16]. Dias et al. [8] reported an increase in yield of AD from tall-oil biotransformation when glycerol was used as carbon source instead of fructose. Borrego et al. [7] also reported that glycerol or fructose increased hydrophobic properties of mycobacterial cell wall but not glucose and using glycerol resulted in the production of cells that have about 40 % thinner cell wall compared to cells grown in glucose and fructose. In this study, the much thinner cell wall and highest cellular hydrophobicity were observed when employing isopropanol as sole carbon source. Clearly, carbon sources producing cells with thinner cell walls exhibited higher biotransformation efficiency. As a solvent, isopropanol was propitious to enhance the solubility of β -sitosterol in the aqueous medium and accelerate the rate of entry of β -sitosterol into the cytoplasm. It indicated the presence of isopropanol to some extent destroyed the rate-limiting step of this biocatalysis reaction for the low solubility and intake of β -sitosterol.

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