The Inhibitory Effect of Cyclodextrin on Oxygen Bioavailability Is a Key Factor for the Metabolic Flux Redistribution Towards Steroid Alcohols in Phytosterol Resting Cells Bioconversion

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Original Article

Keywords: hydroxypropyl-β-cyclodextrin, Mycobacterium, oxygen bioavailability, steroid alcohol, redox balance

Posted Date: February 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-196986/v1

Version of Record: A version of this preprint was published at Applied Biochemistry and Biotechnology on March 13th, 2021. See the published version at https://doi.org/10.1007/s12010-021-03540-w.

Abstract

In the present work, we tried to identify the mechanism why by which the steroid alcohols accumulated when hydroxypropyl-β-cyclodextrin (HP-β-CD) was present to enhance the sterol conversion rate. Compared with the bioconversion system without HP-β-CD, the reaction rate was greatly improved in presence of HP-β-CD, but the steroid alcohols largely accumulated concurrently. The concentration of steroid alcohols increased with the enhanced reaction rate and the higher intracellular NADH/NAD^+ level was detected. *Mycobacterium neoaurum* mutants with higher KshA activity (3-ketosteroid 9α-hydrolase, a monooxygenase hydroxylating the nucleus at C-9 at the expense of NAD(P)H consumption) reduced the steroid alcohols production and in the meantime, the NADH/NAD^+ level was decreased consequently. Further research found that oxygen availability was seriously inhibited by the cyclodextrin in a reaction system. These results indicated that NADH formed in the bioconversion was not properly regenerated via the respiratory chain because of the poor oxygen bioavailability. The inhibitory effect of cyclodextrin on oxygen bioavailability is a key factor for the metabolic flux redistribution towards steroid alcohols in phytosterol resting cells bioconversion.

Introduction

In recent decades, the cleavage of sterol side chain by microbes (bacteria and fungi) was widely investigated to produce steroids, such as 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) [1]. The steroid intermediates production is carried out by microbials through the cleavage of the side chain of steroids at C-17, which is similar to the β-oxidation process of fatty acids [2]. Key genes involved in sterol degradation were identified with the help of genome sequencing and sequence analysis [3, 4]. Furtherly, genome sequence analysis and gene engineering promoted the development of engineered strains for steroids production through bioconversion process [5–7].

One of the reasons that phytosterol bioconversion proceeds slowly was due to the extremely low solubility of sterols in the aqueous phase [2]. To tackle this problem, measures have been used to enhance the bioavailability of substrate, including the use of surfactants, ionic liquids, oil-water emulsification, cyclodextrin [8–10]. Among them, CD, especially HP-β-CD, was reported to be an excellent performance in the enhancement of phytosterols conversion rate mainly by forming the sterol-cyclodextrin inclusion complex [10]. As reported, CDs have multiple functions on the sterol bioconversion, including the potential inhibition of steroid core degradation, the increase of cell wall permeability for both steroids and nutrients to promote the sterol bioconversion or the inhibition of the expression of some genes related to sterol degradation [11–13].

In our previous work, the efficient bioconversion of phytosterols to steroids using engineered *Mycobacterium neoaurum* was achieved through a HP-β-CD-resting cells reaction system [14]. High sterol concentrations and reaction rates showed that the bioprocess of microbial side-chain cleavage of phytosterols for steroids production was useful even in industry. However, we also found by quantitative analysis of individual metabolites, that the steroid C22-alcohols were accumulated during the

bioconversion process. This result was in agreement with the researches of Donova et al [1] but the formation mechanisms are still unknown. During the sterol degradation, NADH ($FADH_2$) and ATP are formed to supply the reducing power and energy for cells growth and metabolism [2]. Many approaches showed that the intracellular redox level in sterol biotransformation played an important role in promoting steroid biotransformation and regulating the metabolic distribution [15, 16].

In this work, the steroidal intermediates production was carried out in a HP-β-CD-resting cells reaction system. The steroid alcohols accumulation in the bioprocess under the condition of different reaction rate were investigated. The possible factors, such as NADH/NAD^+ and dissolved oxygen level, affecting metabolic redistribution leading to the formation of steroid alcohols were discussed. The objective of this research is to clarify the mechanism of steroid alcohols accumulation in the sterol bioconversion.

Materials And Methods

Strain and media

The strain of *M. neoaurum* ATCC 25795 and its mutants used in this study are shown in Table 1. Engineered strains were constructed according to the procedure of reference as previously stated [17].

Table 1 Strains used in this study

M. neoaurum	Description	References
NwIB-R10	NwIB-R10-Δ*kshA*, AD producer.	[17]
NwIB-R10-*kshA*	NwIB-R10-Δ*kshA::kshA*	This study
NwIB-yV	NwIB-yV-pMV261-*kshA*; 9-OHAD producer.	[17]

Preculture medium consisted (per liter) of 0.5 g K_2HPO_4, 0.5 g $MgSO_4$, 2.68 g NH_4Cl, 2.0 g citric acid, 0.05 g ammonium ferric citrate, 20.0 g glycerol. Growth medium consisted (per liter) of 2.0 g citric acid, 0.05 g ammonium ferric citrate, 15.0 g glucose, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$, 3.5 g $(NH_4)_2HPO_4$, 1 g corn steep powder, 0.1 g phytosterol. The initial pH was adjusted to 8.0 using 2 mol L^{-1} NaOH. Media were sterilized at 115°C for 20 min.

Reagents

Phytosterol (purity 95.2%) was purchased from Davi Biochemistry Inc. (Huzhou, China), containing 47.5% β-sitosterol, 17.7% stigmasterol, 26.4% campesterol, 3.6% brassicasterol. AD, ADD and related steroids were purchased from Sigma (St. Louis, USA). HP-β-CD was purchased from RSC Chemical Industries Co. Ltd. (Kunshan, China). Corn steep powder was purchased from Xiwang Group Co. Ltd. (Binzhou, China). Other chemicals were analytic grade reagents from Sinochem. Co. Ltd (Shanghai, China).

Preparation Of Resting Cells And Biotransformation

The seed was incubated in preculture medium at 30°C, 200 rpm for 2 days. Then, 5% (v/v) preculture broth was transferred to growth medium for cell expansion at 30°C, 200 rpm for 3 days. *M. neoaurum* cells were harvested by centrifugation (8000 g) at 4°C for 15 min and the cell pellets were washed twice with 20 mmol L^{-1} phosphate buffer (PB, pH 8.0) and finally resuspended in the same buffer for further use.

All the bioconversion process with resting *M. neoaurum* cells was performed in 20 mmol L^{-1} PB (pH 8.0) under non-sterile conditions in a 250-mL flask at 30°C, 200 rpm, containing a mixture of 1:1 molar ratio of HP-β-CD to phytosterol (approximately 4:1 by weight). The concentration of wet cell pellets (WCW) and phytosterol was based on the requirement of each test. The bioconversion volume (20 mL) was maintained constant by the addition of distilled water each day to offset the evaporation. All the bioconversion of phytosterol was performed in triplicate.

Effect Of Hp-β-cd On Oxygen Availability

Sulfite could react with the oxygen in aqueous reaction system to form sulfate. Effect of HP-β-CD on oxygen availability was determined in a reaction system containing different concentration of HP-β-CD without *M. neoaurum* cells and substrate. The reactions were performed in a 3.7-L bioreactor with 2 L working volume at 30 °C, constant agitation and aeration rate and initiated by adding sodium sulfite (final concentration, 0.5 mol L^{-1}). The oxygen utility rate was detected using exhaust gas analysis apparatus (Shandong Academy of Science, Jinan, China) when the oxygen utility rate was constant in each reaction with different concentration of HP-β-CD.

Analytical methods

The procedure of sample processing and steroids analysis by HPLC were described as reference [18]. The phytosterol was analyzed by HPLC with a Agilent Zorbax SB-C18 (5 μm, 4.6×250 mm, Shanghai, China) at 40 °C and a UV detector at 210 nm. The mobile phase was composed of acetonitrile/isopropanol (70: 30, v/v), and a flow rate of 1 mL min^{-1} was used.

According to the instructions, the level of NADH/NAD^+ at one-day bioconversion was detected by EnzyFluo™ NAD^+/NADH Assay Kit (EFND-100) based on an enzyme-catalyzed kinetic reaction (Beijing Lablead Biotech Co. Ltd., Beijing, China).

Results

Metabolites analysis of bioprocesses with and without HP-β-CD

In order to improve the reaction rate of sterol bioconversion, the HP-β-CD was applied in the reaction system. But the composition of metabolites was different between the reaction system with or without

HP-β-CD. In the reaction system without HP-β-CD, the highest production of AD reached 0.4 g L^{-1} and the substrate consumption was not more than 20% after 3-day bioconversion. Because of low solubility of phytosterols, the reaction rate was greatly limited. Component analysis of metabolites (Fig. 1a) indicated that the AD was the major product, which accounted for about 95% of total metabolites. Minor by-products, such as ADD, 22-hydroxy-23,24-bisnorchol-4-en-3-one (HBC, less than 3%), were also detected in the broth. When the bioconversion process was carried in the medium with the HP-*β*-CD, the conversion rate was greatly improved. The substrate was completely consumed within 2 days. However, the quantitative analysis of individual metabolites showed that much more steroid alcohols, such as HBC, 1,4-HBC, which accounted for more than 35% of total metabolites (Fig. 1b), had been produced during the biotransformation than that of the process without HP-β-CD. These metabolites were uncompleted side-chain degradation products of sterols during the bioconversion and considered as the reduced metabolites (a scheme of sterol catabolism to these metabolites was shown in Supplement Figure). Preliminarily, it was speculated that the increased production of steroid C22-alcohols was related to the higher reaction rate in a reaction system in the presence of HP-β-CD.

Effect of reaction rate on the production of steroid C22-alcohols in M. neoaurum NwIB-R10

To investigate the effect of reaction rate on the production of steroid C22-alcohols, we designed a bioprocess with 50 g/L of substrate and different concentration of biomass. As shown in Fig. 2a, the apparent reaction rate increased with higher cells (catalysts) concentration, while the reaction rate per unit biomass (wet cell weight) decreased, which is the indication of intracellular metabolic flux (Fig. 2b). The substrate was completely consumed except for 25 g/L biomass after 5-day conversion, and higher reaction rate per unit biomass in different reaction systems led to higher production of steroid C22-alcohols (Fig. 2b). In the meantime, the impact of intracellular NADH/NAD^+ ratio on the formation of steroid C22-alcohols was investigated (shown in Fig. 3). The higher reaction rate per unit biomass also led to higher levels of NADH/NAD^+ and thus to redistribution of flux toward steroid C22-alcohols for NAD^+ regeneration putatively.

Comparison of the steroid alcohols level among M. neoaurum mutants with different KshA activity

Ksh of *M. neoaurum* belongs to a Rieske-type oxygenase which can catalyze the hydroxylation at C-9 of steroid nucleus. *Mycobacterium neoaurum* has two KshA homologues, KshA1 and KshA2. The *kshA1* is the key gene involved in the hydroxylation at C-9 of steroid nucleus, and the *kshA2* is a redundant form of *kshA1* [6]. The reaction of 9-hydroxylation requires two molecules of reducing equivalents (NAD(P)H) from the reductase of sterol metabolism to the oxygenase [19]. *M. neoaurum* mutants with different KshA activity were constructed as follows: NwIB-R10 is a *kshA* deletion strain, NwIB-R10-*kshA* is a replenishing strain Δ*kshA::kshA*, and NwIB-yV is a *KshA* over-expressed strain. The results of bioconversion by *M. neoaurum* mutants were shown in Table 2. There were no significant differences in reaction rate with different mutants and the substrate was consumed completely within 5-day bioconversion. No 9-hydroxylated products (9-OHAD, DHBC, DHC) were detected in the sterol bioconversion with strain NwIB-R10 because of *kshA* deletion. The 9-hydroxylated products in the sterol bioconversion with the other

mutants increased with the increase of KshA activity of mutants. *M. neoaurum* NwIB-yV which is a *KshA* over-expressed strain even produced 9-OHAD as major product. The C-9 hydroxylation products level represented the KshA activities of mutants. Interestingly, the ratio of steroid C22/C24-alcohols (HBC, 1,4-HBC, DHBC, DHC) was decreased from 51.3–22.8% when the mutants with different 9-hydroxylation activity of was used to consume surplus NADH formed in sterol bioconversion with HP-β-CD.

Table 2
Metabolites analysis of the bioconversion with different mutants

Strains	Ratio of steroid products (%)[a]							
	9-OHAD	AD	ADD	HBC[b]	1,4-HBC[b]	DHBC[b]	DHC[b]	steroid alcohols
NwIB-R10	ND	44.9 ± 0.2	3.8 ± 0.1	47.7 ± 0.1	3.6 ± 0.3	ND	ND	51.3
NwIB-R10-*kshA*	26.2 ± 1.1	33.5 ± 0.8	3.3 ± 0.2	22.2 ± 0.6	ND	11.9 ± 0.6	2.9 ± 0.0	37.0
NwIB-yV	75.6 ± 2.3	1.6 ± 1.3	ND	Trace	ND	18.0 ± 1.2	4.8 ± 0.2	22.8

Note: a, The ratio of products was determined by quantitative analysis of individual metabolites using area normalization after separation by HPLC. b, denotes the steroid alcohols with the hydroxyl at the end of the side chain. ND, not detected. All tests were performed in a reaction system containing 50 g L^{-1} substrate, 200 g L^{-1} HP-β-CD and 50 g L^{-1} biomass (wet cell weight) of different mutants in 250-mL flasks with 20 mL working volume for 5 days

The detection of NADH/NAD^+ level with different mutants (as shown in Fig. 4) also confirmed the effect of KshA activity on the production of steroid alcohols. As the KshA activity increase of each mutants, the intracellular NADH/NAD^+ level was decreased from 0.21 of strain NwIB-R10 to 0.16 of strain NwIB-yV. Obviously, the increase of C-9 hydroxylation reaction in *M. neoaurum* accelerated the regeneration of NAD^+ and consequently decreased the production of steroid alcohols correspondently (Table 2).

Effect of HP-β-CD on oxygen availability

Generally, the reduced metabolites were more inclined to be formed in the anoxic conditions [20]. Therefore, it was assumed that the presence of HP-β-CD possibly led to oxygen shortage in the sterol bioconversion. As shown in Fig. 5, the oxygen utility rate decreased with the increasing concentration of HP-β-CD and was only 29% of the control when the HP-β-CD concentration reached 25 g L^{-1}. Using dextrin instead of HP-β-CD to keep the same viscosity of reaction system, there was only little effect on the oxygen utility rate. Because the chemical reaction of oxygen and sodium sulfite is not a rate limiting step, it indicated that the oxygen transfer rate was decreased in the presence of HP-β-CD due to formation of inclusion complexes. For developing an industrial bioprocess, high substrate concentration (50 g L^{-1} or more) was applied. Addition of HP-β-CD in proportion to the hydrophobic phytosterols improved the bioavailability of substrate by the formation of stable inclusion complexes. Apparently, this gave rise to

the application of high concentration of HP-β-CD (200 g L^{-1}) in the reaction system. The bioprocess of sterol degradation would therefore proceed under conditions of limited oxygen availability, determined by the oxygen transfer rate. Obviously, the presence of HP-β-CD was the main factor to affect the oxygen consumption rate. The limited oxygen availability in a reaction system hampered the regeneration of NADH and led to redistribution of metabolic flux to the steroid alcohol in sterol conversions.

Discussion

M. neoaurum is an efficient producer of steroids by side-chain degradation of sterols under aerobic condition. Although the application of HP-β-CD-resting cells reaction system improved the sterol bioconversion by solubilizing the hydrophobic substrate, it also affected the oxygen bioavailability of reaction system and then the intracellular metabolism. It is estimated that the side-chain removal of one molecule of β-sitosterol yields 21 molecules of NADH and 10 molecules of $FADH_2$ [2]. To maintain the carbon catabolism, NADH produced from the microbial metabolism must be recycled to NAD^+ for ATP generation via the respiratory chain. However, the regeneration activity of NAD^+ and FAD via respiratory chain was inhibited because of the limited oxygen bioavailability in the presence of HP-β-CD. To achieve the redox balance, the formation of the steroid alcohols is an alternative way to reoxidize NADH to NAD^+ when the activity of respiratory chain is not high enough. Such a self-balance in the cofactor system of microbes can maintain functional stability and dynamic homeostasis in a redox state automatically, to adapt to the changing environment [20, 21]. Flux redistribution is expected in organisms in which the steroid alcohols are formed to regenerate NAD^+. Results also showed that a higher cellular kshA activity can accelerate NADH regeneration, and contribute to less production of steroid alcohols.

Microbial sterols side-chain degradation is initiated by C26 (or C-27) hydroxylation which is catalyzed by cytochrome P450 coupled to the consumption of oxygen and then followed by further oxidation to the sterol C26-oic acid and the cleavage of alkyl sterol side chain at C-17 via the similar fatty acid β-oxidation process [22]. Badejo et al reported a molecular adaptation to a hypoxic mode of respiration during aerobic pyrene degradation by *Mycobacterium gilvum* PYR-GCK [23]. With the high affinity of the aromatic ring cleavage oxygenases for molecular oxygen, the oxygenase activity is more competitive compared to the cytochrome oxidase activity. That means the reaction rate of side-chain degradation has been less affected than the regeneration of NAD^+ via the respiratory chain despite of the oxygen shortage in the bioprocess of sterols metabolism caused by oxygen transfer rate limitation. It will burden the regeneration of NAD^+ and finally lead to the formation of steroid alcohols.

The last step of side-chain degradation at C-17 will require the participation of a hydroxyacyl-CoA lyase like other CoA-lyases, not by a conventional β-oxidation due to the presence of the cyclopentane ring D [24]. In addition, the reaction rate of acyl-SCoA dehydrogenase or enoyl hydratase right near to the steroid skeleton is lower than at the terminal part of the side-chain of sterol due to the steric hindrance effect [2]. This is perhaps the reason why steroid C22-alcohols are accumulated in the process of phytosterol degradation when the intracellular metabolic flux change.

This work demonstrated the putative mechanism of bisnorcholene derivatives accumulation in sterol bioconversion when the cyclodextrin was present to enhance the conversion rate. The redox imbalance caused by increased NADH production and oxygen shortage in the bioprocess is the main reason of the steroid alcohol's formation. The shift of metabolic flux toward steroid alcohols is the intrinsic property of redox self-balance of *M. neoaurum* metabolism. These approaches gave an insight into metabolic flux regulation to target products via biotechnological approaches reshaping the whole-cell response to redox balance and will help the rational development of industrial bioconversion strains or processing strategies.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Acknowledgements

This work was supported by National Natural Science Foundation of China (31570079, 21276083).

Author Contributions

Xue-Dong Wang designed the experiments. Kuan Chen and Dan-Dan Cao performed experiments. Xue-Dong Wang and Dong-Zhi Wei analyzed the experimental data and wrote the manuscript. All authors discussed the results and commented on the manuscript at all stages.

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Figures

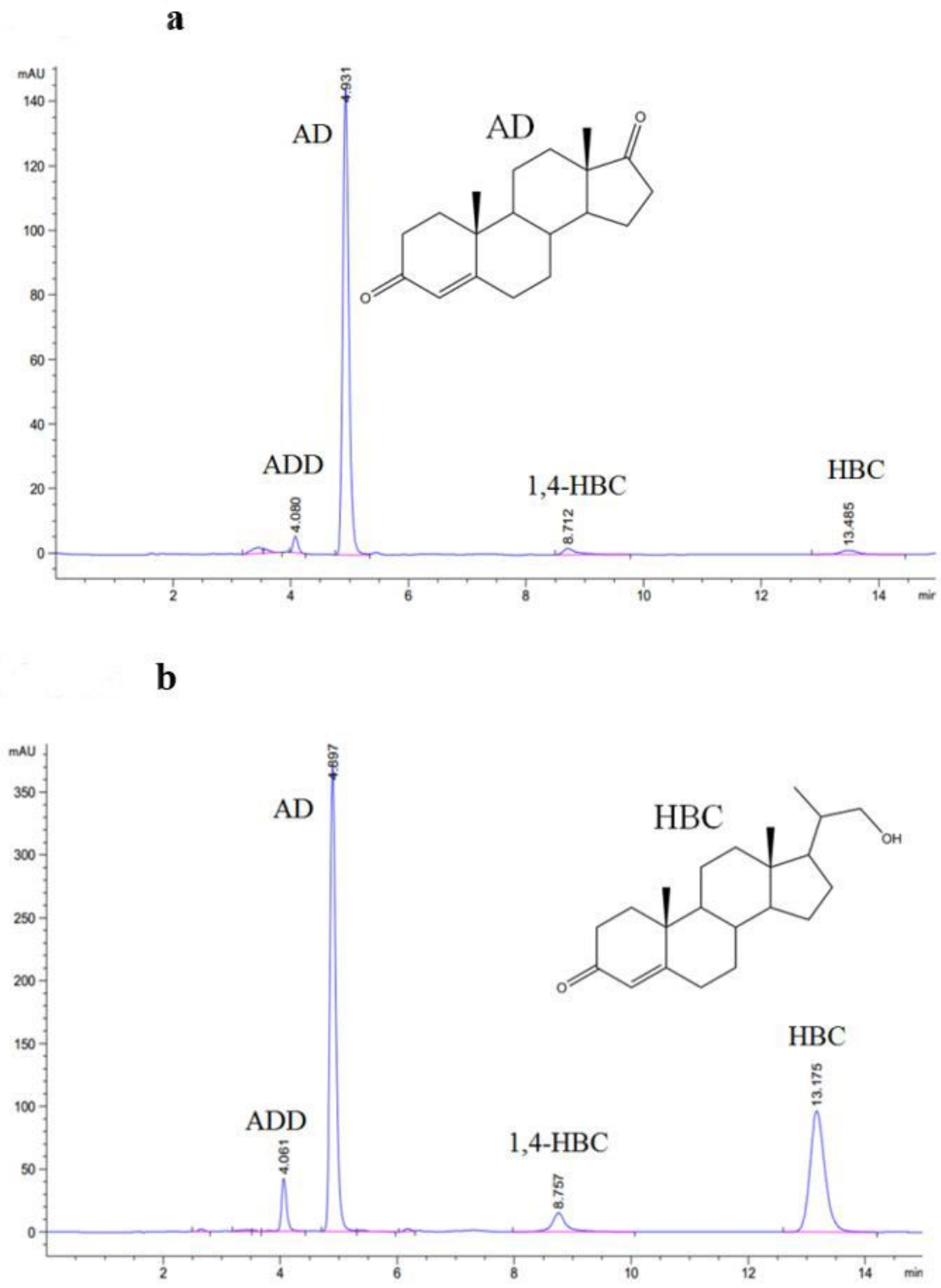

Figure 1

HPLC chromatograms of the metabolites in different reaction systems. The bioconversions in a reaction system (a) with 40 g L-1 and (b) without HP-β-CD, containing 50 g L-1 biomass (wet cell weight), 10 g L-1 of phytosterol, were carried out with M. neoaurum NwIB-R10 resting cell in 250-mL flasks with 20 mL of reaction volume for 3 days

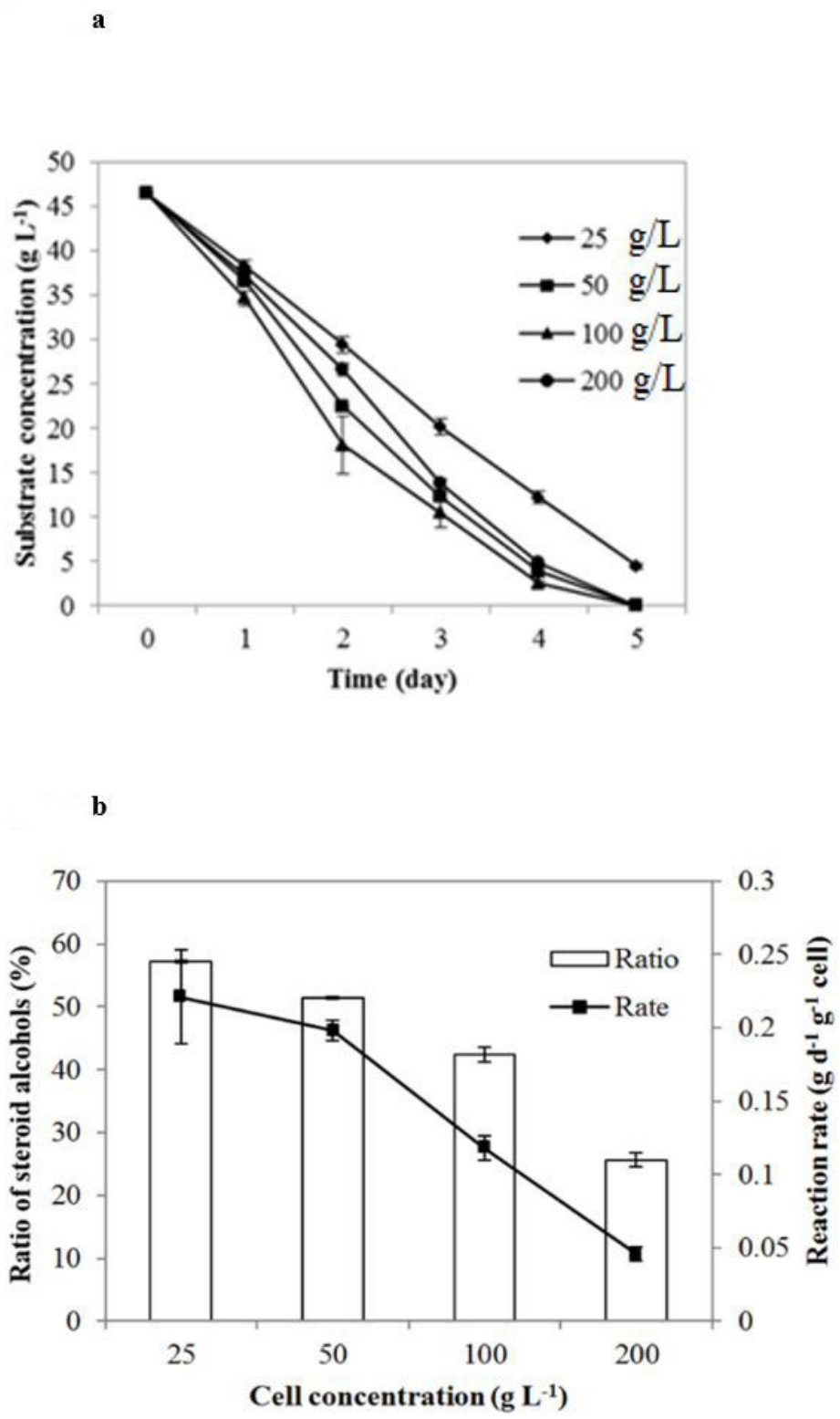

Figure 2

Effect of the reaction rate on the production of steroid alcohols. a, substrate profile; b, reaction rate per unit biomass (wet cell weight) and ratio of the steroid alcohols. All tests were performed in a reaction system containing 50 g L-1 substrate, 200 g L-1 HP-β-CD and 25-200 g L-1 biomass (wet cell weight) in 250-mL flasks with 20 mL working volume for 5 days. The substrate consumption over 24 h was

assumed as the initial reaction rate. The ratio of steroid alcohols was determined by the quantitative analysis of individual metabolites using area normalization after separation by HPLC

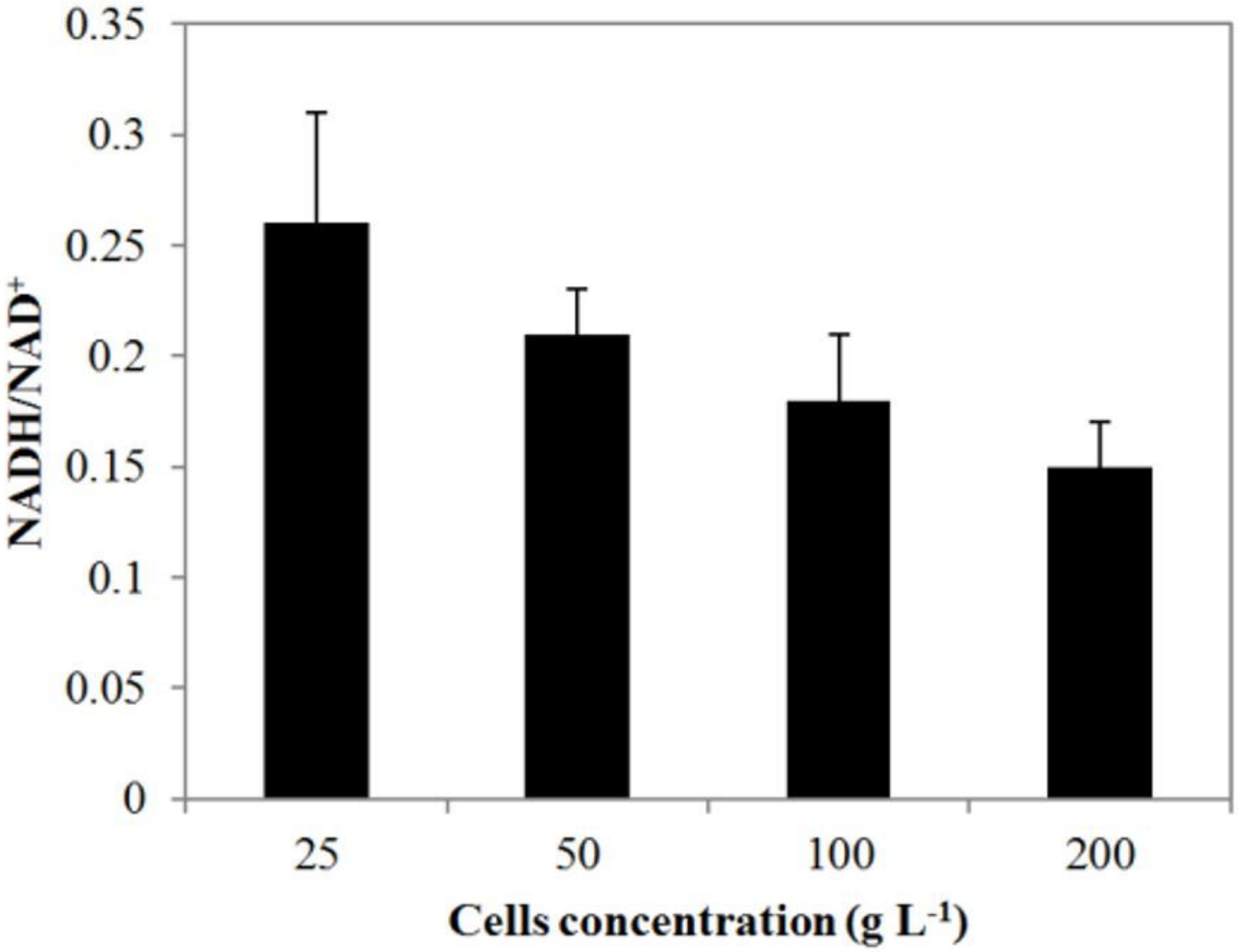

Figure 3

NADH/NAD+ level in a reaction system with different cell concentration. There was a significant difference between groups ($p < 0.01$). Intracellular NADH/NAD+ level was determined at 1-day bioconversion

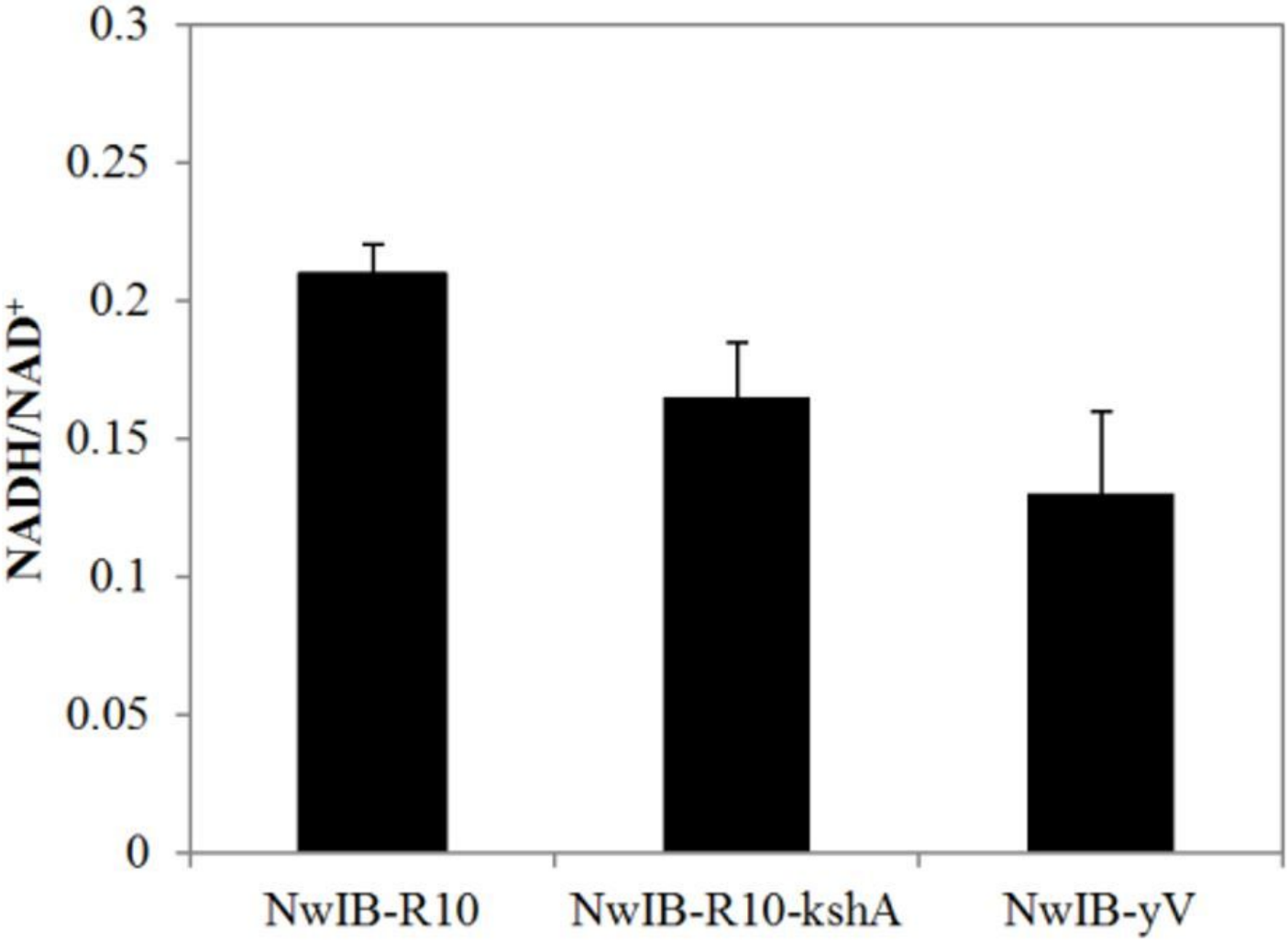

Figure 4

NADH/NAD+ level in a reaction system with different mutants. There was a significant difference between groups ($p < 0.01$). All bioconversion was performed in a reaction system containing 50 g L-1 substrate, 200 g L-1 HP-β-CD and 50 g L-1 biomass (wet cell weight) of different mutants in 250-mL flasks with 20 mL working volume for 5 days. Intracellular NADH/NAD+ level was determined at 1 day bioconversion

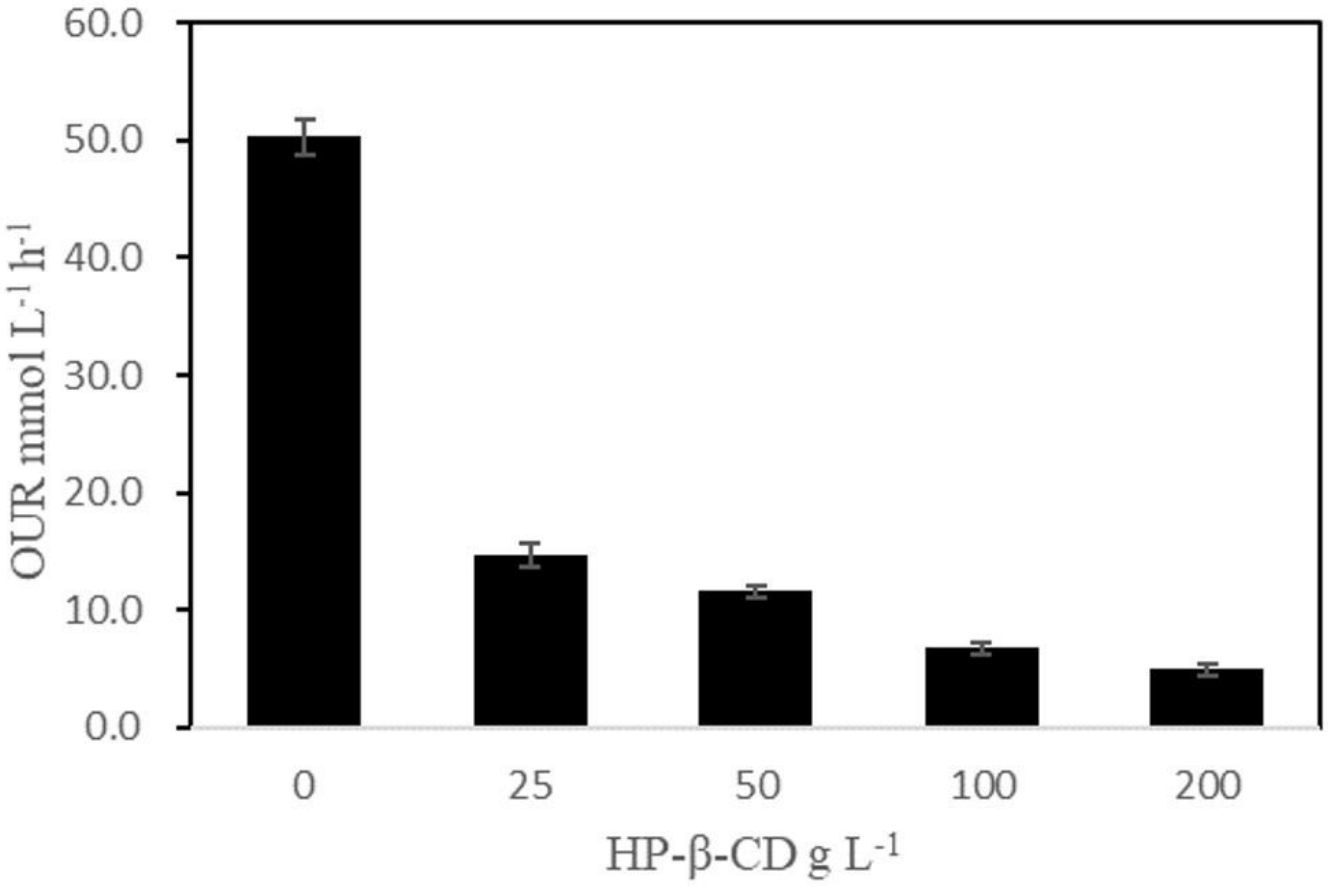

Figure 5

Effect of HP-β-CD on oxygen utility rate

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementFigure.docx