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Biotransformation of various natural sterols to androstenones by *Mycobacterium* sp. and some steroid-converting microbial strains

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Abstract

The selected standard bacterial strains (*Arthrobacter citreus* TISTR 820, *Bacillus sphaericus* ATCC 13805, *Bacilllus stearothermophilus* TISTR 329, *Corynebacterium aquaticum* TISTR 823, *Pseudomonas acidovorans* TISTR 356, *Streptomyces peucetius* TISTR 3355) and fungal strains (*Aspergillus niger* TISTR 3254, *Aspergillus terreus* TISTR 3109, *Cunninghemella elegans* TISTR 3370, *Curvularia lunata* TISTR 3292, *Penicillin siamensis* TISTR 1253, *Rhizopus arrhizus* TISTR 3188) were screened for sterol side-chain cleavage activity using β-sitosterol as a model substrate. In various solubility conditions, the main predicted products, 4-androstene-3,17-dione (AD), 1,4-androstadiene-3,17-dione (ADD) and testosterone (TS) were not obtained in all screening species. Both of *Mycobacterium* sp. NRRL B-3683 and *Mycobacterium* sp. NRRL B-3805 were able to convert β-sitosterol to AD and ADD. The principal product from *Mycobacterium* sp. NRRL B-3683 was AD, meanwhile *Mycobacterium* sp. NRRL B-3805 was ADD. Structural feature of sterol substrates affected to androstenone production. Compared to all tested sterols, β-sitosterol was higher converted into total androstenones in yield of 75.87 and 83.86% by *Mycobacterium* sp. NRRL B-3683 and *Mycobacterium* sp. NRRL B-3805, respectively. Almost equivalent of maximum AD and ADD with total conversion of 81.83% was observed in mixed cultures of both strains.

Keywords: Biotransformation; *Mycobacterium* sp.; Sterol; 1,4-Androstadiene-3,17-dione; 4-Androstene-3,17-dione

1. Introduction

Comparing to chemical synthesis methods, the transformation of steroids by microbiological methods is of considerable commercial interest [1]. Microbial degradation of the side chain of natural sterols has received much attention in the pharmaceutical industry [2,3]. The steroidal intermediates from mentioned reaction can be used for the synthesis of all type I aromatase inhibitors [4,5] and other several high-value steroidal drugs [2,6]. The bioconversion step is the degradation of the C-17 side chain of steroid without concomitant degradation of the steroid nucleus [7,8]. The development of economically feasible processes based on these sterol results both from their low cost with abundantly available and from the ease of their transformation into steroid intermediates [9]. Sterols are a group of naturally occurring substances contained a total of 27–30 carbon atoms in which a side chain with carbon atoms ≥7 is attached at C-17. They are of similar structures only due to the variations of the number and location of double bond and to the number of carbon atoms composing the side chain [10]. The vast majority of microbial side-chain degradation reaction is still based mainly on cholestane-based compounds of both animal and plant origin, such as cholesterol and phytosterol mixtures, sitosterols [6]. The phytosterol compositions may be obtained from a by-product of pulp and paper industry [11]. These substrates possess relatively long, mostly fully saturated, side-chains of 8–10 carbon atoms. By contrast with β-sitosterol (phytosterol) and cholesterol, two natural sterols with unsaturated side-chain, stigmasterol and ergosterol has not been widely applied as a substrate for the biotransformation. In addition, the selective sterol side-chain cleavage is much more limited in both bacteria and fungi being confined mainly to the various species of *Mycobacterium* genus

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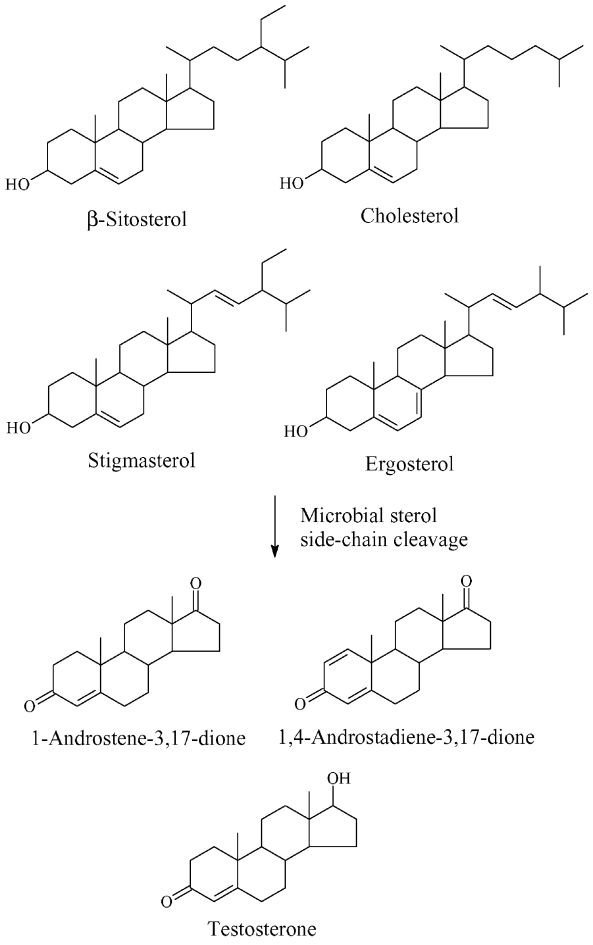

Fig. 1. Sterol side-chain cleavage reaction.

[2,3]. Various methods of adding sterol substrates to fermentation media for bioconversion were performed [2,11–17].

The propose of this study was to screening the selected bacterial and fungal strains for sterol side-chain cleavage activity using 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) and testosterone (TS) (Fig. 1) as the possible end-products of the biotransformation. For attempt to increase solubility of sterol, the biotransformations were carried out in various conditions using water-miscible solvent and surfactants. As far as biotransformations by *Mycobacterium* sp. are concerned, we have not found in the literature any reports on the correlation between a sterol substrate structure and the transformation profile. For this reason we have chosen for our research 4 substrates with different side chains, i.e. β-sitosterol, cholesterol, stigmasterol and ergosterol (Fig. 1). The androstenone productions by *Mycobacterium* sp. NRRL B-3683, *Mycobacterium* sp. NRRL B-3805 and mixed strains were also evaluated.

2. Experimental

2.1. Chemicals

β-Sitosterol (60%) and cholesterol (99%) obtained from Fluka (Buchs, Switzerland) and stigmaterol (95%) and ergosterol (75%) obtained from Sigma (St. Louis, MO) were used as substrates for biotransformation. 4-Androstene-3,17-dione, 1,4-androstenediene-3,17-dione and testosterone were reference grade from Sigma. Yeast extract (Fluka), Tris–HCl buffer (Fluka), D-(+)-glucose (Riedel-de Haen, Seelze, Germany) were used for biotransformation media. Tween 80 (BDH, Poole, England), Dioctyl phthalate (Aldrich, Milwaukee, WI) and Triton X-114 (Fluka) were used for improved steroid substrate solubility. All other chemicals and solvents used were of reagent grade and were procured from various standard sources.

2.2. Microorganisms

The standard bacterial strains were: *Bacillus sphaericus* ATCC 13805 (American Type Culture Collection, ATCC, Manassas, VA); *Arthrobacter citreus* TISTR 820, *Bacillus stearotherophilus* TISTR 329, *Corynebacterium aquaticum* TISTR 823, *Pseudomonas acidovorans* TISTR 356 and *Streptomyces peucetius* TISTR 3355 (Thailand Institute of Scientific and Technological Research, TISTR, Pratumthanee, Thailand); and *Mycobacterium* sp. NRRL B-3683 and *Mycobacterium* sp. NRRL B-3805 (German National Resource Centre for Biological Material, DSMZ, Braunschweig, Germany). The standard fungal strains, *Aspergillus niger* TISTR 3254, *Aspergillus terreus* TISTR 3109, *Cunninghemella elegans* TISTR 3370, *Curvularia lunata* TISTR 3292, *Penicillium siamensis* TISTR 1253 and *Rhizopus arrhizus* TISTR 3188 were from TISTR. The organisms were kept in the form of revival freeze-dried culture. The bacterial and fungal cells were reactivated by culturing on nutrient agar, tryptic soy agar or potato dextrose agar slants at room temperature.

2.3. Screening of microbial cells for sterol side-chain cleavage

Stock cultures of microbial strain kept on agar were subcultured in primary seed culture medium (50 mL) containing ($g\,L^{-1}$): yeast extract, 5; ammonium sulfate, 3; and magnesium chloride in 50 mM Tris–HCl buffer pH 7.8 for bacterial cells and glucose, 10; corn steep liquor, 8; and yeast extract, 2 for fungal cells in 250-mL shake flask held at room temperature, 200 rpm on the orbital shaker (Revco Scientific Inc., Asheville, NC) for 2 days. All aseptic techniques were done in laminar air flow cabinet (Forma Scientific Inc., Marietta, OH). After cultivation, a 1.0 mL of seed medium was added to the biotransformation medium (50 mL) containing the same medium above. β-sitosterol was vigorously mixed with either solvent or surfactant prior use and then a mixture of 5–20 mg β-sitosterol in various conditions such as 95% ethanol (4% v/v), Tween 80 (1 and 8% w/v), dioctyl phthalate (2% v/v) and Triton X-114 (1% w/v) was added to the biotransformation media after 2 days of cultivation. The reaction flask was held at $25 \pm 2\,^{\circ}C$, 200 rpm on the rotary shaker for 3 days. The total of biotransformation media collected at 0–3 days were analyzed by HPLC.

2.4. Biotransformation of sterols by Mycobacterium sp.

Stock cultures of *Mycobacterium* strain kept on agar were subcultured in primary seed culture medium for bacterial cells and followed by biotransformation medium in the conditions as described in Section 2.3. After 2 days of cultivation, a mixture of each 10 mg sterol substrate (β-sitosterol, cholesterol, stigmasterol and ergosterol) in Tween 80 (8% w/v) was added to the biotransformation media. The reaction flask was controlled as conditions in Section 2.3 for 14 or 18 days. The total of biotransformation media collected at 0, 2, 5, 7, 9, 12, 14, 16 and 18 days were analyzed by HPLC.

2.5. Biotransformation of sterols by mixed culture of Mycobacterium sp.

Stock cultures of each *Mycobacterium* strain kept on agar were subcultured in primary seed culture medium for bacterial cells in the conditions as described in Section 2.3. After 2 days of cultivation, 0.5 mL of each seed medium was added to the biotransformation medium containing the same medium above. After 2 days of cultivation, a mixture of β-sitosterol (10 mg) in Tween 80 (8% w/v) was added to the biotransformation media. The reaction flask was controlled as conditions in Section 2.3 for 18 days. The total of biotransformation media collected at 0, 2, 5, 7, 9, 12, 14, 16 and 18 days were analyzed by HPLC.

2.6. HPLC analysis

At the end of the incubation period, the total media taken from the flask was extracted with 50 mL ethyl acetate by vigorously mixing for 10 min in separatory funnel. After separation, the organic layer was evaporated and dried. The residue was then dissolved in 1.0 mL mobile phase. A 100 μL sample diluted with 400 μL mobile phase was filtered through 0.45 μm nylon syringe filter and transferred to a sampling vial. Samples (100 μL) were analyzed by HPLC consisted of a solvent delivery system (Varian 9012, Varian, Palo Alto, CA) and a variable wavelength UV–Vis detector (Varian 9050, Varian) equipped with a Rheodyne (7725) sample injector (Rohnert Park, CA) fitted with a 100-μl sample loop. The chromatographic separations were carried out on HyperClone C-18 column (250 mm × 4.6 mm i.d.; 5 μm particle diameter, 250 Å average pore size) (Phenomenex, Torrance, CA) using 45% water, 40% acetonitrile and 15% methanol as the mobile phase at a flow rate of 1.0 mL min^{-1} and a UV–Vis detector (238 nm) was used. The retention time of authentic reference samples, ADD, TS and AD were 6.3, 7.0 and 8.5 min, respectively. For qualitative study, the retention time of biotransformation products in the chromatograms were compared to AD, ADD and TS peaks. Calculations of obtained weight of androstenones were determined by the calibration curve of authentic reference samples using the area under the curve of biotransformation products in each chromatogram. Conversion of sterol into AD, ADD and TS was estimated as follows:

$$\text{conversion}\,(\%) = \frac{[\text{weight androstenone}/\text{MW androstenone}] \times 100}{[\text{weight sterol}/\text{MW sterol}]}$$

where MW is the molecular weight [18].

3. Results and discussion

3.1. Screening of microbial strains for sterol side-chain cleavage

Extensive works have been mainly performed regarding the selective side-chain cleavage of phytosterols into steroid intermediates such as AD, ADD and TS by *Mycobacterium* sp. [2,3,19,20]. In mammalian reaction the conversion of cholesterol to pregnenolone during progesterone biosynthesis was catalyzed by cytochrome P450 [6]. The side-chain cleavage of sterols in microorganisms was involved various catabolic enzymes and this process consisted of 14 consecutive enzymatic steps [21]. However, literature data on the microbial side-chain cleavage while maintaining the steroid nucleus intact from novel biocatalysts are limited. Only few strains, *Mycobacterium* sp., *Lactobacillus bulgaricus* and *Moraxella* sp. were found [2]. In this study, the preliminary screening for the sterol side-chain cleavage by using microbial strains which has never been reported for this activity was done. Six standard bacteria: *A. citreus*, *B. sphaericus*, *B. stearothermophilus*, *C. aquaticum*, *P. acidovorans* and *S. peucetius*, and six standard fungi: *A. niger*, *A. terreus*, *C. elegans*, *C. lunata, P. siamensis* and *R. arrhizus* were selected for this biotransformation study, according to their reports in ability to transformation of steroids and related compounds. According to the previous common works of the sterol side-chain cleavage led to the selection of β-sitosterol as a phytosterol substrate. Due to the low water solubility of the sterol substrate, β-sitosterol was added into the culture medium by dissolving in various solubilizing agents and nonionic surfactant i.e. ethanol, dioctyl phthalate [22], Triton X-114 [15], Tween 80 [15] or their combination, which had been successfully used for transformation of β-sitosterol by *Mycobacterium* sp.

Most of biotransformations of sterols were carried out by growing cells. Comparing the retention time of the HPLC chromatograms obtained from biotransformation products to the predicted androstenones (AD, ADD and TS), no conversion products were observed in all experiments when β-sitosterol were incubated in the presence of either bacterial or fungal strains. The consequences of this study indicated that these mentioned microbial cells were not capable to degrading the side-chain of sterols. Many bacterial genera were capable of utilizing steroids as a sole carbon and energy source, thereby degrading steroids completely to carbon dioxide and water [9,23]. Also, no biotransformation product may caused by the characteristic activity of enzymes in these microbial cells. Furthermore, there were many reasons that β-sitosterol was not converted to the products by microorganisms. The insoluble substrate appeared to be the main problem of the biotransformation. Solvents that

allowed a high solubility strongly interfered with the biocatalytic functions [2]. To prove these mentioned solubility problems, further attempted experiments were performed by a model system using the selective cleavage of the β-sitosterol side-chain by free *Mycobacterium* sp. cells.

3.2. Biotransformation of sterols by Mycobacterium sp.

The specific side-chain cleavage of sterols to AD and ADD, using two species of *Mycobacterium*, were studies. When comparing to the retention of authentic reference samples, only two products, AD and ADD were observed in both *Mycobacterium* sp. NRRL B-3683 and *Mycobacterium* sp. NRRL B-3805. No formation of TS from these sterols was detected. The oxygen supply, NADH and using mutant strains seemed to be the important parameters for the 17β-reduction of AD to TS [24]. Also, 9α-hydroxy-4-androstene-3,17-dione, a metabolite product from the mutant strain of *Mycobacterium* sp. [25], was not observed. The time course of sterol substrates (β-sitosterol, cholesterol, stigmasterol and ergosterol) effect on the AD and ADD production during the bioconversion runs are presented in Tables 1 and 2 for *Mycobacterium* sp. NRRL B-3683 and *Mycobacterium* sp. NRRL B-3805, respectively. The numbers of both culture cells slowly increased (data not shown) comparing to general bacteria. Almost of the experiments, the formation of product was started after 5 days of incubation. Thus, the microbial transformation was presumably related to cell growth. A similar result was also obtained by Wang et al. using the growing cell biotransformation [15]. However, the biotransformation of sterols into AD and ADD by resting cell has been also achieved in cloud point system [16,17]. The fermentation period can vary from about 6–25 days, depending in part on the quantity of phytosterols to be converted to the end products [11]. Long-term continuous cultivation (day 14–18) of both strains using a wide variety of sterol substrates provided an increasing productivity, but the bacterial cells seem to be lost of viability. The overall conversion rate was relative slow compared to other published data [15] since the experiments were done in the different nutrient media and conditions. Effect of the substrate structures on the total production of androstenones (AD and ADD) was compared in Fig. 2. Maximum conversions of sterols were summarized in Fig. 3. β-Sitosterol presented the highest AD and ADD productivity in both strains. Phytosterols (60% purity of β-sitosterol) generally contained with other sterols such as campesterol and sitostanol, which can also be converted to androstenones, may affect to its high accumulated conversion. Also, low content of β-sitosterol in added substrate sample can easier soluble in the medium comparing to other sterols. In all sterols, the side-chain cleavage activity from *Mycobacterium* sp. NRRL B-3805 was higher than *Mycobacterium* sp. NRRL B-3683, according to the total androstenone production. Strain of *Mycobacterium* sp. NRRL B-3683 actively converted all selected sterols to AD as a major product. In contrast, *Mycobacterium* sp. NRRL B-3805 was shown to form ADD higher than AD, except when using cholesterol as a substrate, thus indicating a dominant in $\Delta^{1,2}$-dehydrogenase activity or loss of 1-ene reductase activity. These results of major product in each strain were not in agreement with that obtained by Perez et al. [18] for *Mycobacterium* sp. NRRL B-3683 and Cruz et al. [14] for *Mycobacterium* sp. NRRL B-3805. The bioconversion of phytosterols utilizing *Mycobacterium* sp. MB 3683 can yielded either AD or ADD as major product depending on their collections [11]. An imbalance in the whole steroid content indicated the possible destruction of the steroid skeleton due to the sterol-transforming

Table 1
Conversion of β-sitosterol, cholesterol, stigmasterol and ergosterol to androstenone products by *Mycobacterium* sp.

Products	Time (day)	Conversion (%)			
		β-Sitosterol	Cholesterol	Stigmasterol	Ergosterol
AD	0	0.00	0.00	0.00	0.00
	2	0.00	<0.01	0.00	0.00
	5	0.00	<0.01	0.00	0.00
	7	47.88	1.21	5.40	15.42
	9	55.12	11.61	12.97	18.65
	12	51.06	26.12	22.84	31.30
	14	63.23	31.25	28.82	32.22
	16	–	–	33.28	31.95
	18	–	–	34.15	35.36
ADD	0	0.00	0.00	0.00	0.00
	2	0.00	0.00	0.00	0.00
	5	0.00	<0.01	0.00	0.00
	7	3.40	<0.01	<0.01	0.46
	9	5.06	<0.01	0.15	0.46
	12	3.50	0.07	1.67	1.58
	14	12.64	1.29	2.18	1.21
	16	–	–	2.76	1.12
	18	–	–	3.34	0.74

NRRL B-3683 ($n=3$). AD, 4-androstene-3,17-dione; ADD: 1,4-androstenediene-3,17-dione.

Table 2
Conversion of β-sitosterol, cholesterol, stigmasterol and ergosterol to androstenone products by *Mycobacterum* sp.

Products	Time (day)	Conversion (%)			
		β-Sitosterol	Cholesterol	Stigmasterol	Ergosterol
AD	0	0.00	0.00	0.00	0.00
	2	0.00	<0.01	0.00	0.00
	5	0.00	<0.01	4.18	0.00
	7	10.62	0.07	7.28	0.83
	9	15.93	0.81	4.32	7.11
	12	14.96	14.11	5.69	7.57
	14	32.92	21.67	4.39	7.48
	16	–	–	10.01	7.66
	18	–	–	11.67	8.59
ADD	0	0.00	0.00	0.00	0.00
	2	0.00	0.27	0.00	0.00
	5	0.00	0.00	9.43	0.00
	7	13.03	0.00	10.01	<0.01
	9	25.47	<0.01	6.53	8.74
	12	50.94	15.02	12.41	18.88
	14	50.94	18.01	9.58	25.01
	16	–	–	18.36	29.29
	18	–	–	30.18	31.43

NRRL B-3805 ($n=3$). AD, 4-androstene-3,17-dione; ADD: 1,4-androstenediene-3,17-dione.

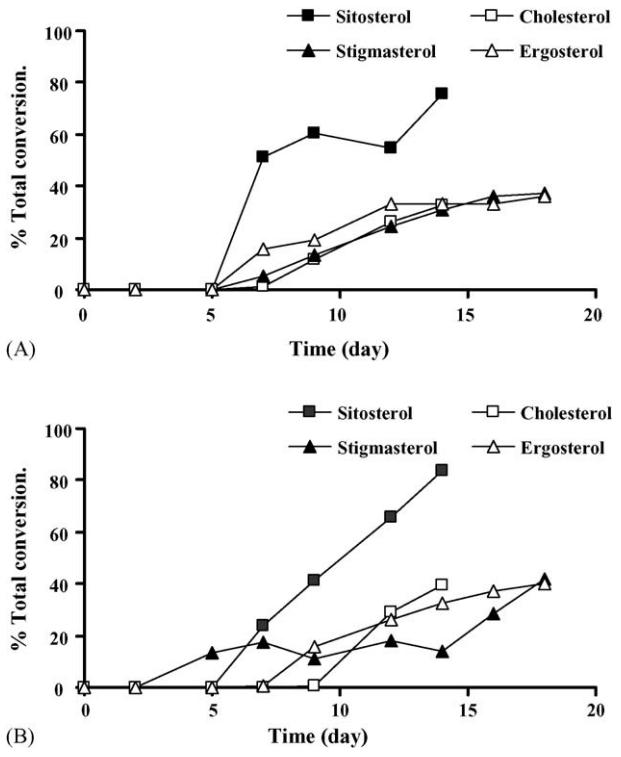

Fig. 2. Time course of the mean total androstane formation with free *Mycobacterium* sp. NRRL B-3683 (A) and *Mycobacterium* sp. NRRL B-3805 (B) using various sterols as substrate ($n = 3$).

activity of unknown enzymes. Generally, a major obstacle in the biotransformation of sterols is the poor solubility of substrates in aqueous system [2]. In this study, β-sitosterol gave higher productivity than other sterols although its solubility was lower regarding to their log *P*-values (β-sitosterol, 8.14; cholesterol, 7.39; stigmasterol, 7.82; and ergosterol, 6.93) predicted by ChemDraw Ultra version 9.0 (CambridgeSoft Corporation, Cambridge, MA). It might be indicated that androstenone production preferably relate to structural feature of substrates rather than the solubility. Additionally, the different conversion rates of these sterols depend on the ability of enzymes to interact with the sterol substrates. As shown in the pathway of cholesterol degradation by *Pseudomonas* sp. [26], acid intermediates (pregn-4-en-3-one-20-carboxylic acid and pregna-1,4-dien-3-one-20-carboxylic acid) were produced in the microbial degradation process to AD or ADD. However, both of them may be not easily formed in case of sterols containing C_{22}–C_{23} double bond in side chain. Moreover, hydrogenation of double bond between C-7 and C-8 in the transformation of ergosterol to androstanes was observed in both strains which similar to the previous report with *Mycobacterium phlei* [27].

Table 3
Production of androstenones from β-sitosterol by mixed culture of *Mycobacterium* sp. NRRL B-3683 and *Mycobacterium* sp.

Time (day)	Conversion (%)		
	AD	ADD	Total
0	0.00	0.00	0.00
2	0.00	0.00	0.00
5	0.00	0.00	0.00
7	27.34	17.86	45.20
9	32.45	15.44	47.89
12	50.55	10.86	61.41
14	44.60	19.55	64.15
16	42.29	18.82	61.11
18	32.69	48.63	81.32

NRRL B-3805 ($n = 3$). AD, 4-androstene-3,17-dione; ADD, 1,4-androstenediene-3,17-dione; Total: sum of AD and ADD.

3.3. Biotransformation of β-sitosterol by mixed culture of *Mycobacterium* strains

As shown in Table 3, the conversions of β-sitosterol were started after 5 days and no other products were formed by mixed growing *Mycobacterium* sp. cells. The times to reached maximum yield in both products were different. For mixed cultures, concomitant lower maximum conversions of AD and ADD when

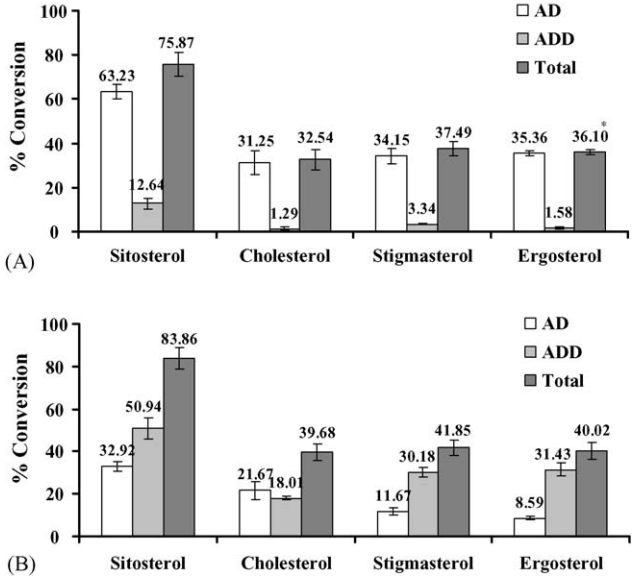

Fig. 3. Effect of sterol substrates on the mean maximum conversions of androstenone products by *Mycobacterium* sp. NRRL B-3683 (A) and *Mycobacterium* sp. NRRL B-3805 (B) (AD: 4-androstene-3,17-dione; ADD: 1,4-androstadiene-3,17-dione; total: sum of AD and ADD; *: the time to reached maximum conversion of AD and ADD was different) ($n = 3$).

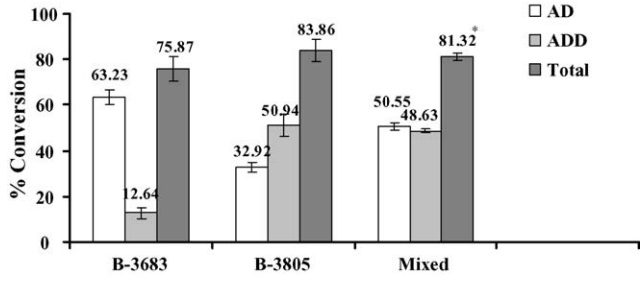

Fig. 4. Comparison of mean maximum conversions of androstenone products by *Mycobacterium* sp. NRRL B-3683, *Mycobacterium* sp. NRRL B-3683 and mixed both strains using β-sitosterol as a substrate (AD: 4-androstene-3,17-dione; ADD: 1,4-androstadiene-3,17-dione; total: sum of AD and ADD; *: The time to reached maximum conversion of AD and ADD was different) ($n = 3$).

comparing to the maximum yield of each isolated strain, were achieved and the maximum of total androstenones was between from both of single strains (Fig. 4).

4. Conclusion

The preliminary screening of the microorganisms leading to the increased availability of new and robust biocatalysts suited for selective cleavage of the side-chain of natural sterol. In this study, no androstenone metabolites determined by HPLC were obtained that may cause by lack or deficiency of specific activity of enzyme from limited screening bacterial and fungal strains. In addition, attempt to enhancing mass transfer of sterol substrate in aqueous media by using non-toxic surfactants was used in various conditions. According to the previous reports of side-chain cleavage microorganism, *Mycobacterium* sp. was selected to prove the biotransformation condition. The molecular structure of natural sterol effected on the productivity of AD and ADD using free *Mycobacterium* sp. strains. The major product obtained from both strains was not significantly identical. The culture media with *Mycobacterium* sp. NRRL B-3805 resulted in greater conversion of sterols to the total metabolites than another one. Comparing to the sterol substrates using for the biotransformation, the results indicated that β-sitosterol was higher converted to total products rather than another type of substrates. For biotranformation of β-sitosterol by mixed culture *Mycobacterium* sp., the maximum conversions of AD and ADD were nearly identical.

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