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ORIGINAL PAPER

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Bioconversion of phytosterols to androstanes by mycobacteria growing on sugar cane mud

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Abstract Direct sterol conversion of sugar cane mud (residue) by *Mycobacterium* sp. was demonstrated to be possible technologically, thus avoiding sugar cane oil extraction and further processes of extraction and purification of phytosterols from this oil. Indeed, mycobacterial cells were able to convert phytosterols from sugar cane mud into 4-androstene-dione (AD) and 1,4 androsta-diene-3,17-dione (ADD). For the various concentrations assayed, concomitant higher yields for both androstanes were achieved at 20% (w/w) sugar cane mud in media. Furthermore, conversions were similar to those from other substrates, such as a mixture of phytosterols. The results suggest that the mycobacterial cell is able to easily access and bioconvert sugar cane mud phytosterols.

Keywords Sugar cane mud · Mycobacteria · Steroids · Bioconversion

Introduction

Microbial conversion of natural sterols (cholesterol or phytosterols) into steroidal precursors has long been used for the synthesis of steroidal drugs of biomedical importance [9, 12]. These include the production of several higher-value steroidal compounds derived from 4-androstene-dione (AD) and 1,4 androsta-diene-3,17-dione (ADD), such as progestational, adrenocortical, estrogenic and contraceptive agents.

The worldwide market for AD and ADD is estimated to be approximately U.S. $ 750×10^6 annually [5]. Processes become cheaper according to lower costs for obtaining such raw materials or substrates [3].

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Byproducts from different industries, e.g., tall oil in the paper industry and mud (residue) in the sugar cane industry can be employed as raw materials for producing phytosterol mixtures. Sitosterol from tall-oil is microbiologically converted to steroid intermediates in yields comparable to the use of pure sitosterol [21]. For instance, Forbes Medi-Tech has developed an innovative fermentation technology that converts plant sterols, isolated from tall oil [10], into pharmaceutical fine chemicals, essential for the production of various pharmaceutical steroids such as contraceptive agents and anti-inflammatories.

Mud from the sugar cane industry contains, among other compounds, a phytosterol mixture of campesterol (25%), β-sitosterol (42%) and stigmasterol (33%) [22]. This sugar cane mud or "cachaza" is otherwise inexpensive and constitutes a serious pollutant. Traditionally, it is used as a fertilizer [24].

The wax after extraction of sugar cane mud may be used to produce a phytosterol-rich oil. Various methods for further extraction and purification of phytosterol mixtures from this oil have been developed and patented [15, 22]. Also, successful microbial fermentation of sugar cane phytosterols to steroid intermediaries has been reported [6, 18].

The overall process of obtaining sugar cane phytosterols is cumbersome and time-wasting, so direct fermentation of the mud might be much cheaper and therefore affordable. The results of direct fermentation of mud by mycobacterial cells may support such an assumption.

Materials and methods

Microorganisms

Mycobacterium sp. strains NRRL- B3683 and MB3683 were used throughout this work [13, 14]. They convert sterols mainly into ADD and AD, respectively.

Media

NB medium [23] [containing, per liter: 8 g nutrient broth (Oxoid), 1 g yeast extract (Oxoid), 10 g glycerol (Merck)] was used for growing *Mycobacterium* cells. To avoid the aggregation of growing cells, 1 g Tween 80 (Merck) was also added, and for solid media, 20 g/l Agar technical no. 3 (Oxoid).

MS medium consisted of various salts and molasses as reported [2].

Bioconversion protocol

Mycobacterial cells were grown in NB medium for 48 h incubation at 30°C with 200 rpm shaking. From cultures with an optical density (at 540 nm) of 1.0, 10% (v/v) were transferred to 50 ml NB or MS medium containing either finely powdered sugar cane mud (10%, 20%, w/v) or a mixture of phytosterols (1 mg/ml) as the substrate for bioconversion. The mud was directly added to the media, while the phytosterol mixture was previously suspended in 1% Tween 80. After 5 days incubation at the above temperature and shaking, cultures were autoclaved and submitted to further chemical analysis. Conversion of substrates into AD or ADD was estimated as follows:

$$\text{Conversion}(\%) = \left[(\text{Weight AD(D)}/\text{Weight of substrate}) \times (\text{MW substrate}/(\text{MW AD}(D)))\right] \times 100$$

where MW is the molecular weight.

Since β-sitosterol represents the greater amount and molecular weight in the phytosterol mixture, it was taken for calculating conversion, both for the phytosterol mixture and the sugar cane mud.

Chemical analysis of products

All cultures were extracted with ethyl acetate. Samples were spotted onto silica gel 60 thin-layer chromatography (TLC) plates and then run under an elution mixture of ethyl acetate:hexane (2:3, v/v). Chemical analysis were carried out by HPLC [18], using a RP-8 column with a flux of 1.5 ml/min, in a methanol:water mixture (65:35). Detection was done at 254 nm. Methyl testosterone was added as an internal standard.

Statistical analysis

In order to support the results statistically, samples were submitted to the classic Student's *t*-test for comparison of media.

Results

Identification of bioconversion products

After extraction of culture broth, TLC revealed AD and ADD as the main bioconversion products, according to strain phenotype. R_f values corresponded to those of pure patterns.

Moreover, HPLC chromatograms (Fig. 1a,b,c) show a coincidence of retention times at 254 nm for both compounds in samples and patterns, which strongly suggests that a proper bioconversion from phytosterols to androstanes took place. Further physico-chemical and spectroscopic assays (data not shown) also supported these results.

In the case of strain B3683, the presence of a peak prior to ADD (Fig. 1c, retention time 1 min) is also remarkable and might represent an acid intermediate of partial phytosterol degradation.

Determination of AD and ADD as bioconversion products

The results of experiments clearly suggest that both strains properly bioconvert the phytosterols from sugar cane mud (Table 1). That is, strain B3683 was able to transform phytosterols mainly to ADD, meanwhile MB3683 did it to AD. The experiments were done at 10% and 20% (w/v) sugar cane mud in both NB and MS media.

A distinctive behavior according to medium and mud concentration was noticed. For both strains, the best results were obtained in MS medium, 20% (w/v) mud. Furthermore, ADD yield in B3683 (22.57 mg) was higher than AD in MB3683 (14.82 mg).

Comparison of AD(D) conversion from direct cachaza and phytosterol mixture

As pointed out before, β-sitosterol (42%), campesterol (25%) and stigmasterol (33%) comprise the total sugar

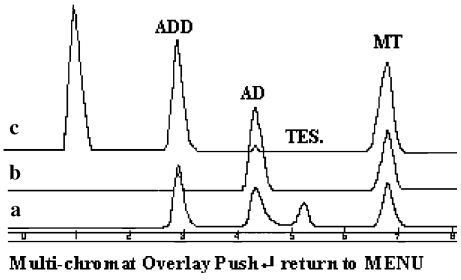

Fig. 1 Chromatogram from supernatant media after sugar cane mud bioconversion. HPLC using a RP-8 column with a 1.5 ml/min flux, in a 65:35 methanol:water mixture. Detection was done at 254 nm. *AD*: 4-androstene-dione, *ADD:* 1,4 androsta-diene-3,17-dione, *TES:* Testosterone. Methyl testosterone (*MT*), as the internal standard for quantification. (**a**) Line showing retention times for steroid patterns, (**b**) strain MB3683, (**c**) strain B3683

Table 1 Direct bioconversion of sugar cane mud by strains of *Mycobacterium* sp. Experiments were carried out in 50 ml MS + substrate (10%, 20%, w/v), for 5 days incubation at 200 rpm, 30°C. Each figure represents the average of three independent determinations ± standard deviation

Strain	Medium	10% mud			20% mud		
		ADD (mg)	AD (mg)	TES (mg)	ADD (mg)	AD (mg)	TES (mg)
B3683	NB	5.33 ± 0.11	5.01 ± 0.31	0.28 ± 0.02	15.80 ± 1.40	4.42 ± 0.81	–
	MS	8.52 ± 0.37	0.32 ± 0.01	–	22.57 ± 2.21	1.28 ± 0.27	–
MB3683	NB	0.32 ± 0.05	8.51 ± 0.05	0.04 ± 0.00	0.29 ± 0.03	7.30 ± 0.38	–
	MS	0.78 ± 0.01	6.17 ± 0.50	–	1.33 ± 0.14	14.82 ± 1.35	–

Table 2 Bioconversion of sugar cane mud vs phytosterol mixture in *Mycobacterium* sp. Fifty milliliters of MS + 1 mg/ml phytosterol mixture or 20% (w/v) sugar cane mud were used as the substrates (see Materials and methods). Conversion (X%) = [weight of AD(D)/weight of substrate] × (MW substrate)/[MW AD(D)] × 100. Each figure represents the average of three independent determinations. Samples were statistically submitted to the classic Student's t-test for comparison of media, $P < 0.05$

Strain	Conversion% ($X \pm$ SD)			
	Sugar cane mud		Phytosterol mixture	
	ADD	AD	ADD	AD
B3683	82.29 ± 8.06	4.65 ± 0.99	95.38 ± 3.52	0.19 ± 0.02
MB3683	4.85 ± 0.50	53.65 ± 4.90	3.66 ± 0.67	64.17 ± 1.84

cane phytosterols [22]. According to our results, that mixture in mud must be available to mycobacterial cells, to be converted to the androstanes AD and ADD.

The phytosterol total content in sugar cane mud (cachaza) ranges from 0.2% to 0.6% (w/w) [1]. The phytosterol content was 0.4% (w/w) in our experiments. Thus, 40 mg was the amount of substrate available in every assay, so that final conversion to ADD must be 82.29% for B3683 and to AD 53.65% for MB3683.

By contrast, phytosterol mixture conversion was significantly greater in both strains: 95.38% ADD for B3683 and 64.17% AD for MB3683, respectively (Table 2). For both sugar cane mud and phytosterol mixture, marked differences are statistically significant at $P < 0.05$.

Discussion

Sugar cane mud or "cachaza" is a well known byproduct of the sugar cane industry. As a whole, this mud thereby constitutes a serious pollutant and is traditionally used as a fertilizer [24]. Moreover, there are several reports concerning the preparation of composts, alcohol and biogas production by microbial fermentation of "cachaza" [16, 17].

Mud contains a wide variety of chemicals as vegetable residues from sugar cane [1, 24]. These compounds are expected to be free and available to any chemical or biological action after the drastic processes of milling and sugar manufacturing. In fact, such compounds can be chemically found in both free and esterified forms. Particularly, phytosterols are extracted from wax and represents up to 0.6% (w/w) [1], so that in suitable media they can be theoretically transformed by microbial cells.

According to our results (Fig. 1), phytosterols in sugar cane mud can be directly converted by mycobacterial cells into the androstanes AD and ADD. Furthermore, physico-chemical and spectroscopic analysis confirm these findings.

As mentioned earlier, the former peak appearing in the B3683 chromatogram (Fig. 1, line c) might correspond to an acid intermediate of partial degradation of a phytosterol side-chain. The partial degradation of side-chains of sterols and bile acids has been reported to produce 3-oxo-4-pregnene-20 carboxylic acid or its analogues [11]. Also, microbial production of 3-oxo-1,4-pregnadiene-20 carboxylic acids from sitosterol in *Corynebacterium* has been patented [8].

Despite the slight viscosity reached in broth containing 20% (w/v) sugar cane mud with respect to 10% (w/v), both AD and ADD yields were apparently higher at the former concentration (Table 1). Increasing the substrate concentration beyond 20% (w/v) did not seems to give good results, possibly due to a much higher viscosity of media. Besides, shaking and cell–medium exchange of metabolites may be hampered when scaling-up, leading to lower yields, so that additional investigation is needed.

Although direct conversion of sugar cane mud to androstanes was rather lower than phytosterol mixture conversion (Table 2), the above results must be taken into account. Thus, the overall extraction and purification of phytosterols might be avoided, likewise the subsequent use of sterol-solubilizing agents, i.e., Tween, vegetable oils, organic solvents or cyclodextrins, in the bioconversion media [4, 7, 19, 20]. Furthermore, the process can convert an industrial pollutant into drugs of biomedical importance and represents a biological pretreatment of mud to be used as a fertilizer.

As a whole, the costs for AD and ADD production from mud were similar to those from purified phytosterols in our laboratory, but additional costs due to transportation of this "cachaza" must be considered, as proposed for some other related processes [24].

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