Appl Microbiol Biotechnol (2001) 57:385–389
DOI 10.1007/s002530100693

ORIGINAL PAPER

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Selection of *Mycobacterium* sp. strains with capacity to biotransform high concentrations of β-sitosterol

Received: 14 December 2000 / Received revision: 27 March 2001 / Accepted: 13 April 2001 / Published online: 6 September 2001

Abstract In this work, phytosterol-biotransforming strains were selected from *Mycobacterium* sp., using a high concentration of β-sitosterol. The selection was made by culturing the strains in a medium enriched with 14 g β-sitosterol/l as the unique source of carbon. During 2 months, the bacterial cultures were transferred successively. The extraction of the biotransformation products was made with methanol and ethyl acetate. The qualitative and quantitative analysis was made by means of thin-layer chromatography, gas-liquid chromatography (GLC) and GLC-mass spectrometry. Under these conditions, it was observed that after seven transfers, the strains *Mycobacterium* sp. MB-3683 and the *Mycobacterium fortuitum* B-11045 increased their biotransformation capacity from 20% to 64% and from 34% to 55%, respectively. The products in the highest proportion identified for each trial were androstenedione and androstadienedione. The results suggest that the high substrate concentration could be a selective mechanism to obtain strains more efficient in the biotransformation of β-sitosterol into steroidal bases.

Introduction

Production of sexual and cortical hormones is one of the most important processes in the pharmaceutical industry (Imada et al. 1981). Currently, the world-wide significance of this process is the result of the growing demand of the population for the use of steroidal hormones and derivatives (Mahato and Garai 1997).

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Obtaining steroidal bases by means of a microbiological process has advantages in comparison to the traditional chemical process, since it decreases the number of stages and produces less polluting reactants (Mahato and Majumdar 1993). However, the microbial process also has some limitations, such as non-specific degradation of the steroidal molecule (Wovcha et al 1978), the low solubility of sterols in aqueous solution (Haberland and Reynolds 1973) and the inhibition of the process when high substrate concentrations are used (Roy et al. 1991).

To address non-specific degradation, obtaining mutant strains has been the most advantageous approach; and this has been achieved by using chemical mutagens such as *N*-methyl-*N*′-nitrosoguanidine (Carguille and McChensey 1974; Wovcha et al. 1978), or by selecting strains using high substrate concentrations (Seidel and Horhold 1992). As to solubility, oil solvents have been used, such as vegetable oils, Tween 80 (Smith et al. 1993) and dimethyl formamide (Shukla et al. 1992), among others. Adsorption resins have also been used, which are able to retain the biotransformation products and do not interfere with the extraction stage of these compounds (Lee et al. 1993; Liu et al. 1994). In spite of the inhibiting effect mentioned above, it has been possible to obtain strains tolerant to high steroidal substrate concentrations, which has favoured the biotransformation process (Seidel and Horhold 1992).

The objective of this work was to select β-sitosterol-biotransforming strains capable of growing in high substrate concentrations.

Materials and methods

Micro-organisms

Mycobacterium sp. MB-3683 strains (Bombay), obtained from *Mycobacterium* sp. NRRL B-3683 by Dr. Rattan Sood, treated with N-methyl-N′-nitrosoguanidine, and *Mycobacterium fortuitum* NRRL B-11045 strains (Wovcha et al 1978) were used and were kept in a freezer at –70 °C (Jones et al. 1984). Subcultures maintained on Middlebrock 7H10 agar slants at 4 °C were used to prepare the inoculum for each trial in a liquid medium.

Materials

Glucose, yeast extract, nutrient broth, nutritive agar and Middlebrock 7H10 agar were purchased from Merck Química Chilena (Santiago, Chile). The substrate was obtained from an isolated fraction of tall oil, which contained 95% phytosterols, of which 78% was β-sitosterol and the remaining percentage was made up of stigmasterol and campesterol (Conner and Rowe 1975).

Media

For preparation of the bacterial inoculum, the medium contained 10 g yeast extract, 10 ml glycerol, 1.5 g $(NH_4)_2$ HPO_4, 1.3 g $K_2HPO_4 \cdot 3H_2O$, 0.2 g $MgSO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.02 g $ZnSO_4 \cdot 7H_2O$ in 1 l of distilled water. The enrichment medium contained 14 g β-sitosterol, 60 g amberlite XAD-2 (Sigma Chemical Company), 1.5 g $(NH_4)_2HPO_4$, 1.3 g $K_2HPO_4 \cdot 3H_2O$, 0.2 g $MgSO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.02 g $ZnSO_4 \cdot 7H_2O$ in 1 l of distilled water (Seidel and Horlhold 1992). The biotransformation medium contained 14 g β-sitosterol, 60 g amberlite XAD-2, 5 g glucose, 10 g yeast extract, 1.5 g $(NH_4)_2HPO_4$, 1.3 g $K_2HPO_4 \cdot 3H_2O$, 0.2 g $MgSO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.02 g $ZnSO_4 \cdot 7H_2O$ in 1 l of distilled water (Seidel and Horhold 1992).

Selection of strains tolerant to high concentrations of β-sitosterol

The strains were cultured in 50 ml of medium enriched with β-sitosterol, according to the methodology described by Seidel and Horhold (1992). The bacterial inoculum was prepared in a liquid medium and 5 ml (10%) were added to the enrichment medium. The culture was kept at 30 °C and constant agitation (180 rpm) for 7 days, using an Heidolph Unimax 2010 orbital agitator. During 2 months, every 7 days, the strains were transferred to a fresh medium. Additionally, the tolerant strains were cultured in a solid medium to which concentrations of 14 g, 28 g and 56 g of β-sitosterol/l were added.

β-Sitosterol biotransformation

The different strains selected were subjected to biotransformation trials in liquid medium in 500 ml Erlenmeyer flasks. The assays were carried out with 14 g β-sitosterol/l, using a bacterial inoculum corresponding to 10% of the end volume (300 ml.) The cultures were kept at 30 °C with constant agitation (180 rpm) for 7 days.

Extraction of biotransformation products

The extraction of biotransformation products was carried out after sterilising the cultures. These were extracted from the amberlite with 150 ml methanol and 150 ml ethyl acetate. The total extracts were concentrated in a rotary evaporator at reduced pressure and then taken to a final volume of 10 ml.

Analysis and identification of products

Each sample obtained from the biotransformation processes was analysed initially by means of thin-layer chromatography (Shah et al. 1980), followed by gas-liquid chromatography (GLC) in a Varian Star 3400 Cx chromatograph and a HP 5890 GLC-mass spectrometer with a HP 5972 mass detector and HP-5MS column and helium as the carrier gas. The identification and quantitation of the compounds obtained were made by GLC-mass spectrometry and comparison with an internal standard of cholesterol. Compounds were purified by chromatography on a Sephadex column and the structure of the pure compounds was determined by ^{1}H-nuclear magnetic resonance (NMR), using $CDCl_3$ as solvent (Singer et al. 1991; Ambrus et al. 1992), and by infrared (IR) spectrophotometry (Lee et al. 1998). The ^{1}H-NMR and IR spectra were carried out in a RMN Bruker AC 250-P spectrometer at 250 MHz and a Nicolet 550 magno-IR spectrophotometer.

Results

Isolation of strains tolerant to high concentrations of β-sitosterol

After each transfer, the tolerant strains were isolated and cultured in a solid medium, to which different concentrations of β-sitosterol were added (14 g/l, 28 g/l, 56 g/l). Initially, 300 strains were obtained, from which 42 strains capable of growing in a solid medium with

Table 1 *Mycobacterium* sp. strains selected with 14 g β-sitosterol/l in the culture medium. Strains derived from *Mycobacterium* sp. MB-3683 produced mainly 4-androstene-3,17-dione (*AD*) and 1,4-androsta-3,17-dione (*ADD*); strains derived from *M. fortuitum* B-11045 also produced 9-hydroxy-(4-androsta-3,17-dione (*9-OH-AD*)

Strains	Biotransformation products (%)		
	AD	ADD	9-OH-AD
MB-3683(1)	51.2	48.0	0.0
MB-3683(2)	70.4	29.3	0.0
MB-3683(3)	68.5	30.1	0.0
MB-3683(4)	81.8	17.5	0.0
MB-3683(5)	61.2	38.5	0.0
MB-3683(6)	60.0	39.5	0.0
MB-3683(7)	72.3	26.7	0.0
MB-3683(27)	71.0	28.9	0.0
MB-3683(29)	95.8	4.1	0.0
MB-3683(30)	30.7	67.8	0.0
B-11045(31)	39.5	41.4	18.5
B-11045(32)	50.0	48.7	1.3
B-11045(33)	19.5	60.0	18.3
B-11045(34)	31.3	64.5	4.2
B-11045(35)	9.8	80.0	10.1
B-11045(39)	10.0	79.5	9.8
B-11045(40)	5.5	80.7	12.0
B-11045(41)	31.2	57.4	11.1
B-11045(42)	29.5	60.1	10.4

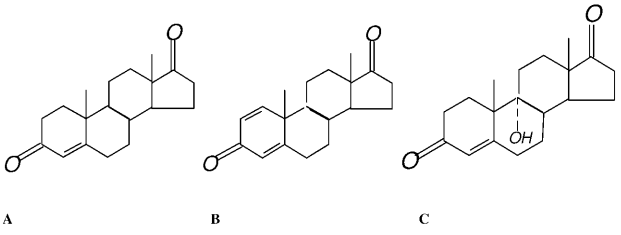

Fig. 1A–C Biotransformation products obtained during the selection process of *Mycobacterium* sp. strains. **A** 4-Androstene-3,17-dione (AD). **B** 1,4-Androsta-3,17-dione (ADD). **C** 9-Hydroxy-(4-androsta-3,17-dione) (9-OH-AD). The wild type only produced AD and ADD; and 9-OH-AD was also produced by the strains derived from *M. fortuitum* B-11045, beginning with the fourth assay

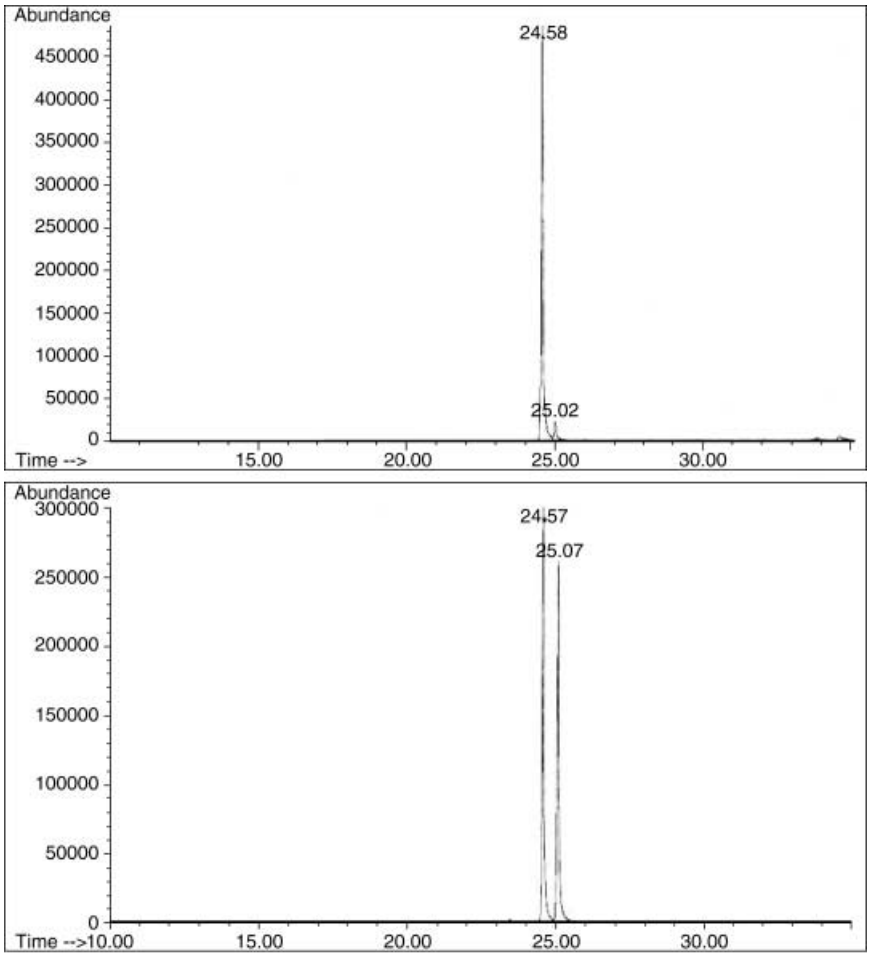

Fig. 2 Biotransformation of phytosterols with different strains of *Mycobacterium* sp. *Top: Mycobacterium* sp. MB-3683 (strain 29); AD retention time 24.58 min, ADD retention time 25.02 min. *Bottom: M. fortuitum* B-11045 (strain 32); AD retention time 24.57 min, ADD retention time 25.07 min

14 g β-sitosterol/l were selected (30 strains from *Mycobacterium* sp. MB-3683 and 12 strains from *M. fortuitum* B-11045). From these strains, 30 grew with concentrations of 28 g β-sitosterol/l and 12 with 56 g β-sitosterol/l. These strains are now part of the culture collection of the Natural Products Chemistry Laboratory of the University of Concepción (Table 1).

β-Sitosterol biotransformation capacity

The 42 strains isolated in the previous stage were subjected to trials in biotransformation cultures, carried out in triplicate in order to determine an average value for their β-sitosterol biotransformation capacity. After seven successive transfers, the *Mycobacterium* sp. MB-3683 strains and the *M. fortuitum* B-11045 strains increased their biotransformation capacity from 22% to 64% and from 34% to 55%, respectively (Table 2).

Table 2 Biotransformation percentages for the selected strains of *Mycobacterium* sp. MB-3683 and *M. fortuitum* B-11045 cultured at a concentration of 14 g β-sitosterol/l. Biotransformation percentages were determined for the wild type and then for each subsequent transfer of the selected strains

Strain	Successive transfers	Biotransformation percentages (%)
Mycobacterium sp. MB-3683	1	22.39
	2	40.28
	3	39.40
	4	46.27
	5	59.54
	6	55.79
	7	64.28
M. fortuitum B-11045	1	34.06
	2	33.10
	3	40.77
	4	45.65
	5	43.35
	6	50.01
	7	55.35

Identification of the products obtained in the biotransformation process

The products obtained in the highest proportions were androstenedione (AD) and androstadienedione (ADD) (Fig. 1A, B). From the 30 strains derived from *Mycobacterium* sp. MB-3683, 20 produced a similar percentage of each product (50%); in the other cases, the main product was AD (Fig. 2A). The 12 strains derived from *M. fortuitum* B-11045 produced mainly ADD (Figs. 2B, 3).

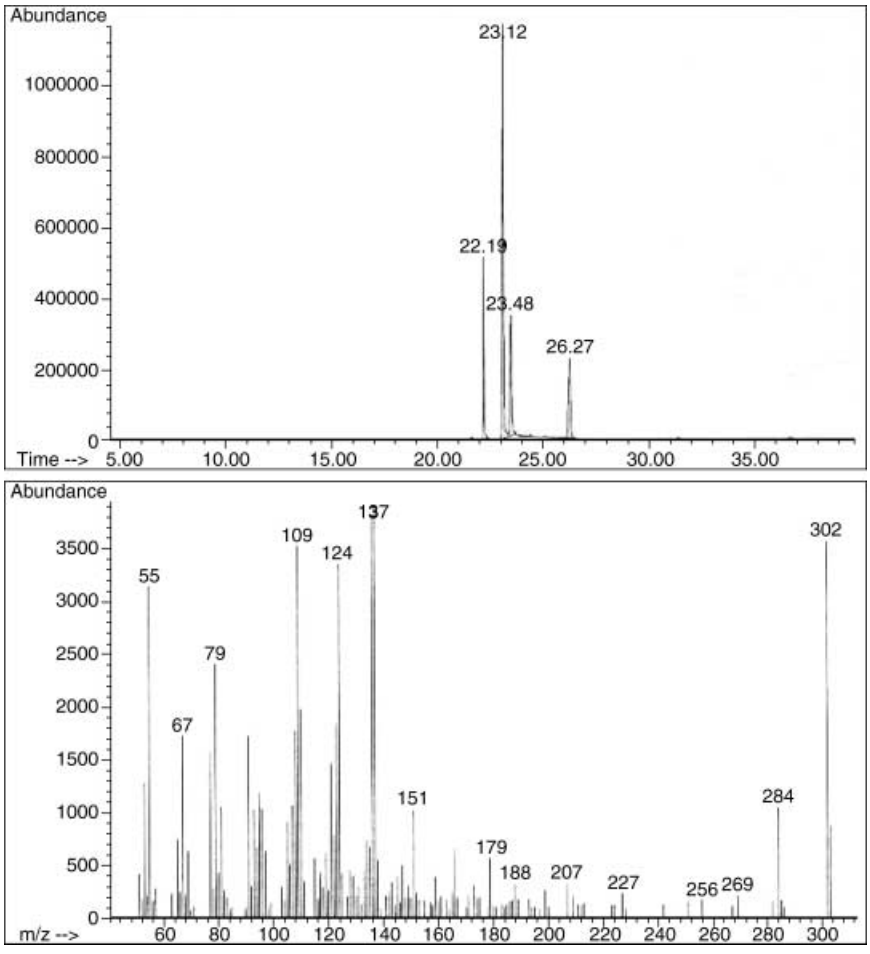

Fig. 3 Chromatogram (*top*) and mass spectral data (*bottom*) for 9-OH-AD. Retention time is 26.27 min and molecular ion value is 302

Discussion

The use of high steroidal substrate concentrations as a selective mechanism for biotransforming strains was excellent in our experiments and is in agreement with the observation of Seidel and Horhold (1992).

In the first transfer, the total biotransformation percentages were less than 35% for the *Mycobacterium* sp. MB-3683 and *M. fortuitum* B-11045 strains. For the MB-3683 strains, the main transformation product was AD and, in the case of the B-11045 strains, the main product was ADD.

From the fourth trial onwards, the biotransformation percentages increased to 50% and the products for the strains derived from MB-3683 were AD and ADD, although some of them began to produce only AD. In the case of the strains derived from B-11045, although the main product was also ADD, an additional new compound corresponding to a hydroxylated AD derivative was also identified (Fig. 1C). At the end of the seventh transfer, biotransformation capacities significantly higher than those at the beginning of the trials were observed; and they exceeded 75% when 14 g β-sitosterol/l were used.

In previous studies, the highest substrate concentrations used in biotransformations of steroidal compounds have been 0.1–1.0 g/l (Roy et al. 1991) and from 1 g/l to 8 g/l (Ahmad and Johri 1993). The strains obtained in our study can tolerate concentrations up to 56 g/l, which indicates that the selection process carried out could be effective in obtaining strains with improved biotransforming activity. The inhibitory effect described by Roy et al. (1991) was observed in early trials, when the strains only biotransformed 20–30% of the substrate. Afterwards, as the strains were transferred, those with higher tolerance to the substrate concentration used (14 g/l) were selected and the biotransformation process was improved.

The production of 9-hydroxyandrostenedione by strains coming from *M. fortuitum* B-11045 could be results of selective pressure, due to the high concentrations of β-sitosterol used. The high concentration of AD could be responsible for the higher enzymatic activity of 9-α-hydroxylase to convert AD into 9-hydroxyandrostenedione. Thus, these strains could be using an alternative pathway in the degradation process of phytosterols. In contrast, the strains coming from *Mycobacterium* sp. MB-3683 could be using the traditional route, but the higher amount of substrate gave a higher yield of the final products. Cellular permeability has not been studied so far and therefore this factor cannot be ignored.

According to our results, the use of high substrate concentrations is an efficient methodology to select strains with phytosterol-biotransforming capacity. In addition, it has the advantage that it can be directed towards obtaining specific characteristics, such as a particular product or a high biotransformation capacity.

Acknowledgements This study was supported by grant FONDEF D-96 1111. The authors also thank the Graduate School of the University of Concepcion, Chile.

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