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SHORT COMMUNICATION

Luffa cylindrica and phytosterols bioconversion: from shake flask to jar bioreactor

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Abstract Bioconversion of lipophilic compounds poorly soluble in water, such as sterols, required the use of chemicals and solubilizing agents. On the other hand, it was shown that immobilization of *Mycobacterium* species on the dried fruit of *Luffa cylindrica* (DFLC) allows a close interaction between immobilized cells and cholesterol particles and increases by then the product's yield. In this work, the use of DFLC in a 5-l jar bioreactor with phytosterols mixture (1 g/l) as substrate was assessed without addition of any chemicals or solubilizing agents. DFLC increased by a factor of four the volumetric productivity of androstenones (0.08 g/l day). Products were accumulated in the aqueous medium while substrates remained on the fibers of DFLC. This observation lets envisage a green semi-continuous process of androstenone production. DFLC has no influence on cell growth, and is moreover natural, inexpensive, non-toxic, and mechanically strong.

Keywords Bioconversion · Phytosterols · Steroids · *Mycobacterium* sp. · *Luffa cylindrica*

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Introduction

Nowadays, steroids represent one of the largest sectors in the pharmaceutical industry with world markets in the region of US$ 10 billion and production exceeding 1,000,000 tons/year [32]. Hormonal steroids are derivatives of androstane, a C19 steroid. Basic building blocks for these important drugs can be derived from natural phytosterols [26], which are the most available as well as being inexpensive raw materials for the production of primary steroids [7]. Development of the selective microbial degradation of sterol side chain without concomitant degradation of the steroid nucleus helped the use of phytosterols as a raw material for androst-4-ene-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD) production [16]. However, the low solubility of sterols in aqueous environment considerably limits the sterol's metabolism by bacteria and reduces then the product's yield [21]. Despite the large amount of experimental data published in scientific and patent literature, the problem of bioavailability of sterol substrates to the biocatalysts still remains to be solved. Methods described in published data include especially the organic-aqueous two-phase system and cloud point system. However, their potential drawbacks are evident. In the organic-aqueous two-phase system, the type and amount of organic solvent must be carefully considered to achieve compatibility with biocatalysts [30]. While in the cloud point system, the key issues are still required (process control and partitioning behavior, particularly when hydrophobic cells are involved) [17].

Whole-cell immobilization has been extensively studied as a means to overcome mass transfer limitation. Cell immobilization offers enhanced fermentation productivity, feasibility of continuous processing, cell stability and lower costs of recovery and recycling and downstream processing

[12]. In previous studies, it has been reported that the dried fruit of *Luffa cylindrica* DFLC is an excellent carrier for algal and plant cells [1, 14], bacterial and yeast cells [11, 23, 25], and hepatocytes of rats [6]. Experiments of cell carrying on DFLC were done for degradation of pesticides [20], removal of some polluting elements in water [2], and producing some chemicals such as ethanol [4, 22], alpha-amylase [11], sorbitol [28], clavulanic acid [25], and L-lactic acid [23].

Furthermore, it has been reported that carrying cells on the dried fruit of *L. cylindrica* (DFLC) increased by 3–4 times the yield of androstenones [24]. Carrying cells on DFLC allows a close interaction between immobilized cells and cholesterol particles and increases by then the products yield. This finding attracted our attention to the use of DFLC for phytosterols bioconversion without use of solubilizing agents. Bioconversion trials of phytosterols were done in shake flasks and in a 5-l jar bioreactor as a first step of scale-up. Hence, the work presented intends to provide a preliminary basis for the use of DFLC in a 5-l jar bioreactor for phytosterols side chain cleavage.

Materials and methods

Microorganism and media

Microorganism strain *Mycobacterium* sp. DSM 2966 was used throughout this study. Tryptic Soy Broth (TSB) was used as a culture and biotransformation medium. TSB is constituted by pancreatic digest of casein (17 g/l), enzymatic digest of soybean meal (3 g/l), dextrose (2.5 g/l), NaCl (5 g/l), and K_2HPO_4 (2.5 g/l) with a pH of 7.3 ± 0.2.

Reagents

Pancreatic digest of casein Bacto Casitone and Enzymatic digest of soybean meal Bacto Soytone-Becton, Dickinson and company (Sparks-USA); sitosterol (at least 40 %), androst-4-ene-3,17-dione (AD), androsta-1,4-diene-3,17-dione (ADD), methanol and acetonitrile were from Sigma-Aldrich (Steinheim, Germany); chloroform (99 %) from Carlo Erba Reactis (Val de Reuil, France). Inorganic salts and other chemicals were all of reagent grade.

Carrying cells

The DFLC was cut into discs approximately 2.5 cm in diameter and 3–4 mm in thickness, soaked in boiling water for 30 min, thoroughly washed under tap water, and left for 24 h in distilled water replaced 3–4 times. The discs were then oven-dried at 70 °C. The adequate amount of DFLC discs was added to the bioconversion medium and autoclaved at 121 °C for 30 min before inoculation with the seed culture.

Bioconversion trials in shake flasks

The strain was grown in two steps. Before each experiment, a preculture was prepared by adding 5 % (vol) inoculum from a 4 °C stored culture to a 30-ml of fresh TSB medium. After 2 days of incubation at 30 °C and 200 rpm, an aliquot of the preculture (5 % vol) was added to bioconversion flasks containing 30 ml of fresh TSB medium and 200 mg of DFLC as cell carriers. The bioconversion flasks were held at 30 °C, 200 rpm for 2 days. The phytosterols mixture (30 mg) was already autoclaved before being added to each flask. Phytosterols proved to be stable after autoclaving. Flasks without DFLC were kept as a control, and the reaction was allowed to proceed for several days. Phytosterols, AD and ADD proved stable in the TSB medium without biocatalysts, under the same conditions as for biotransformation. The total bioconversion media and DFLC were collected at different times for analysis. All experiments were repeated at least three times. All aseptic techniques were done in a laminar air flow cabinet (SKAN AG, VS-120-AFX, Basel, Switzerland).

Bioconversion in jar bioreactor

Bioconversion of phytosterols was carried out in a 5-l jar bioreactor Biostat Aplus (Sartorius, Germany) loaded with 3 l of TSB and 20 g of DFLC. Then, 48-h 5 % inoculum (150 ml) was added and the fermentation was allowed to proceed for 7 days under the following general conditions: temperature of 30 °C, aeration rate of 1 vvm, stirring speed of 200 rpm, and pH of 7.2 ± 0.5. Foaming was controlled by the addition of silicone antifoam M compound (Dow-Corning). Phytosterols mixture (3 g) was already autoclaved before being added as a powder 2 days after inoculation. Aliquots of about 20 ml of bioconversion medium were collected aseptically at different times for analysis.

Growth measurements

Cell growth measurement was done as described by Meyers et al. [19]. Briefly, hot NaOH was employed to release total cellular protein. For each sample, the absorbance was measured at 230 and 260 nm with a Thermo Nanodrop 1000 spectrophotometer, and the protein concentration was determined from the equation (protein) (µg/ml) = $(183 \times A_{230}) - (75.8 \times A_{260})$. Blank measurements for the medium and DFLC without bacteria were also done. The amount of protein was related to dry biomass using an adequate calibration

curve: protein (mg/ml) = 0.359 × cell dry weight (mg/ml) + 0.037 (R^2 = 0.98).

Extraction procedure

Phytosterols and their bioconversion products were extracted from the bioconversion medium by shaking the aqueous samples two times with 1 V of chloroform. Extracts were combined, dried with anhydrous magnesium sulfate, reduced under vacuum to dryness, and the residue was dissolved in chloroform for GC analysis or in chloroform:acetonitrile (2:8) for HPLC analysis.

HPLC analysis

The residue of the extract was dissolved in a chloroform:acetonitrile mixture (2:8) and 1 ml was filtered through 0.2 µm PTFE syringe filter and transferred to a sampling vial. Samples were analyzed by HPLC (Agilent 1100) consisted of a solvent delivery system and a variable-wavelength UV–Vis detector equipped with a sample injector fitted with a 100-µl sample loop. The chromatographic separations were carried out on with an Octyl Ultrasphere C18 column (250 × 4.6 mm i.d.; 5-µm particle diameter, 80-Å average pore size) using the following gradient retention program where the mobile phase flow rate of 1 ml/min: 0.0–0.5 min, water:methanol:acetonitrile 45:27.5:27.5; 0.5 min to 15.5 min linear decreasing of water % in mobile phase with keeping equal % of methanol and acetonitrile; 15.5–25 min, methanol:acetonitrile 50:50. Peak detection was performed at 204 and 238 nm. The retention time of authentic reference samples, ADD, AD, dihydrobrassicasterol, campesterol, and β-sitosterol were 7.0, 8.2, 20.5, 21.3, and 22 min, respectively (Online resource 1).

GC-MS analysis

The GC-MS analyses were carried out on a Shimadzu GCMS-QP 2010 using a BP-X5 column (25 m × 0.15 mm i.d. × 0.25 µm). Helium was used as a carrier gas. The injector and detector temperatures were 225 and 310 °C, respectively. Helium was used as carrier gas (20 psi). The temperature program began at 225 °C for 0.5 min and proceeded firstly at 1 °C/min to 280 °C, and secondly at 20 °C/min to 310 °C with a final hold at 310 °C for 15 min. The injection was carried out in splitless mode at 225 °C. The MS was operated in the electron impact mode at 70-eV ion source energy, with source temperature at 200 °C in scan mode m/z = 40–500. The mass spectra were compared with those of the NIST 05 database and authentic standards (Online resource 2–6).

Scanning electron microscopy

Samples of DFLC with immobilized cells were washed with deionized water and were then oven-dried at 105 °C for 24 h. Samples were covered with a gold film and observation was done on a Philips XL-30 FEG scanning electron microscope.

Results and discussion

In a previous work, promising results were obtained with DFLC used as cell carrier in androstenones AD(D) production from cholesterol with growing *Mycobacterium* sp. cells [24]. The feasibility of using DFLC was tentatively addressed with a phytosterols mixture (β-sitosterol: campesterol: dihydrobrassicasterol = 56:30:13, molar ratio) used as substrate and added to the medium as a fine powder.

Figure 1a and b show typical biotransformation kinetics obtained in shake flasks without or with DFLC, respectively. Campesterol and dihydrobrassicasterol were consumed equally by *Mycobacterium* sp. DSM 2966 since their conversion rates after 7 days were about of 18 and 20 % respectively (Fig. 1a). Consumption of β-sitosterol was clearly lower with a conversion rate of 9 % (Fig. 1a). Several works report that the abilities of bacteria to transform sterols into 17-ketosteroids decrease in the following order: cholesterol > campesterol > β-sitosterol > stigmasterol > ergosterol [29]. This preferential consumption is also observed in the presence of DFLC in the shaken flasks: the conversion rates of β-sitosterol, campesterol, and dihydrobrassicasterol were about of 41, 57, and 61 %, respectively (Fig. 1b).

Adding DFLC dramatically increased AD(D) accumulation as compared to the control (Fig. 1). In control experiments, the final amounts of accumulated AD and ADD were about of 7.9 and 33.4 mg/l, respectively. In flasks containing DFLC, 58.6 mg/l of AD and 249.1 mg/l of ADD were detected. Similarly, growth measurements showed that a higher biomass was obtained when using DFLC. In the later case, the amount of total cells TC was divided equally between suspended cells (SC) and immobilized cells (IC) (Fig 1). However, the higher bioconversion rates observed with DFLC cannot just be explained by the higher biomass. Indeed, the specific productivity of AD(D) in flasks with DFLC (0.0198 $g_{AD(D)}/g_{dry\ biomass} \times$ day) show that a nearly fivefold increase has occurred as compared to the control (0.0039 $g_{AD(D)}/g_{dry\ biomass} \times$ day).

Scanning electron microscopy of DFLC fibers taken after 2 days of incubation showed adequate retention of cells (Fig. 2). Generally, cells with hydrophobic properties prefer material with hydrophobic surfaces for adhesion,

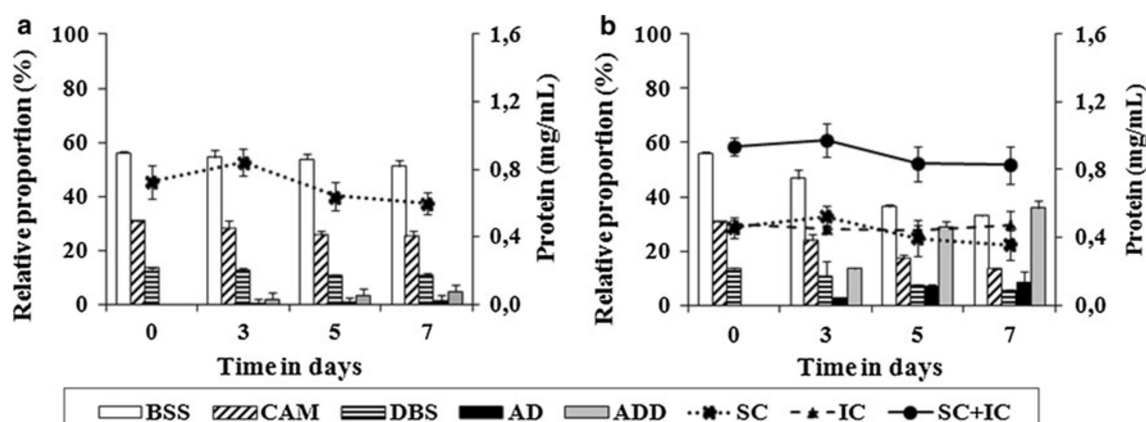

Fig. 1 Bioconversion of phytosterols mixture (1 g/l) by *Mycobacterium* sp. DSM 2966 in shake flasks (100 ml) without (**a**) and with (**b**) DFLC (200 mg) in TSB medium. The operating conditions were: working volume, 30/100 ml; inoculum size, 5 %; temperature, 30 °C; agitation speed, 200 rpm. *BSS* β-sitosterol, *CAM* campesterol, *DHB* dihydrobrassicasterol, *SC* suspended cells, *IC* immobilized cells, and *TC* total cells

and those with hydrophilic characteristics prefer hydrophilic surfaces. The cell wall of *Mycobacterium* has been reported to have high hydrophobicity [15] and DFLC to contains hydrophobic lignin (15 %) [8]. Furthermore, the irregularities of material surfaces promote bacterial adhesion in contrast to ultrasmooth surfaces [18]. In this way, the surface roughness of DFLC and its hydrophobic domains allowed the passive adhesion of *Mycobacterium* cells. The exact counting of immobilized cells was practically difficult due to cell aggregation in clumps. If it was one cellular layer, the cell density could be estimated around 1.4–1.8 cell/μm^2.

This adequate adhesion of mycobacterial cells to DFLC coupled to its high specific area lead to a tridimensional network of biocatalysts fulfilling the medium and increasing by then the interaction between cells and sterol particles. The direct contact is essential for sterol uptake by cells [3]. This observation explains the higher accumulation of AD(D) in DFLC flasks.

Given the promising results obtained in shake flasks, the production of AD(D) from the phytosterols mixture was carried out in a 5-l jar bioreactor, an experimental set-up more amenable for scale up. As shown in Fig. 3, relative dissolved oxygen level decreased to near zero at 48 h of cultivation and then increased accompanied by increased accumulation of AD(D) in the medium. This typical "V"-shaped curve has been reported [9, 10]. The decrease of relative dissolved oxygen was explained by cell growth and sterols bioconversion. Indeed, the sterol side chain degradation involves oxidation reactions that required dissolved oxygen in the medium. Maintaining a constant relative dissolved oxygen level required oxygen supplying system or stirring speed increasing which can damage cell integrity. However, it was shown previously that the yield of the bioconversion process is depressed by the overaeration of

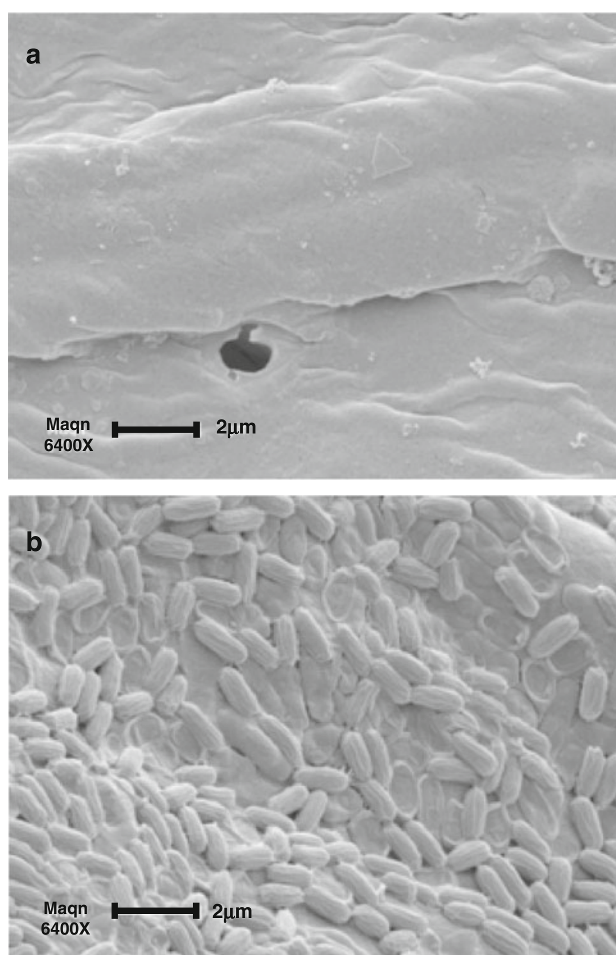

Fig. 2 Scanning electron micrographs of DFLC fibers without (**a**) and with (**b**) immobilized *Mycobacterium* sp. DSM 2966

the reaction mixture [27]. In contrast to relative dissolved oxygen, cell growth increased with oxygen consumption and then decreased after 5 days of cultivation in both

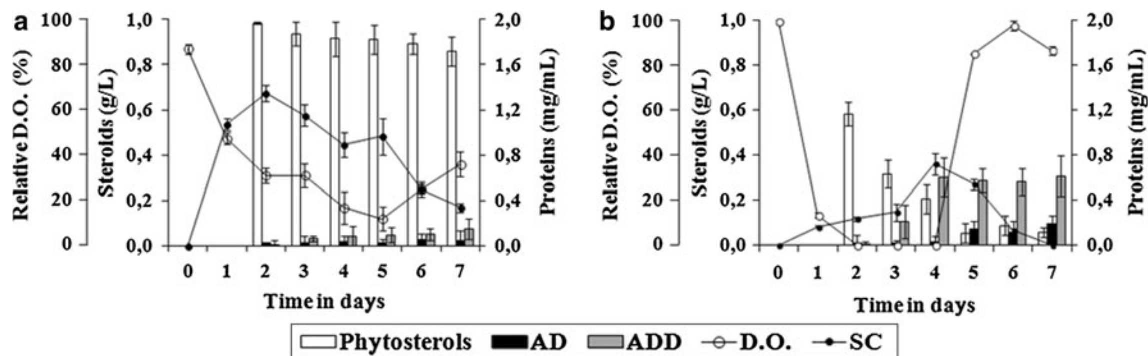

Fig. 3 Production of androstenones from phytosterols mixture by *Mycobacterium* sp. DSM 2966 in a 5-l jar bioreactor without (**a**) and with 20 g DFLC (**b**). The operation conditions were: working volume, 3/5 l TSB medium; inoculums size, 5 %; temperature, 30 °C; stirring speed, 200 rpm; aeration rate, 1 vvm; pH, 7.2 ± 0.5; phytosterols mixture, 1 g/l added 2 days after inoculation. *DO* dissolved oxygen, *SC* suspended cells

control and DFLC experiments (Fig. 3). However, in the latter case, it was practically impossible to remove DFLC aseptically with the aliquots. Thus, protein measurements for DFLC experiments (Fig. 3b) represent the amount of suspended cells without that of immobilized cells. At the end of the experiment, the total amount of DFLC (20 g) was removed for analysis and the protein content of immobilized cells was about 3.92 mg/ml in the medium (i.e., 0.53 $g_{\text{dry biomass}}/g_{\text{DFLC}}$).

Sterols tend to be waxy and do not undergo milling very well; they also tend to clump and do not disperse well in the medium [13]. As a consequence, most sterol particles adhere to cells immobilized on DFLC, forming stable agglomerates ("substrate immobilized cells") [3]. This phenomenon explains the low sterol content in aliquots (0.6 g/l) at day 2 instead of the theoretical 1 g/l. Therefore, the results showed in Fig. 3b represent the steroid composition of medium aliquots and not the overall composition in the bioreactor. The ADD accumulation in the medium reached a maximum of about 0.30 g/l at day 4 with a yield of about of 43 % (Fig. 3b) compared to an ADD yield of 11 % at day 7 in trials without DFLC (Fig. 3a). The volumetric productivity of both AD(D) was about 0.08 g/l.day with DFLC compared to 0.018 g/l.day in control experiments. Higher volumetric productivities of AD were obtained previously with liquid polymer-based systems (0.572 g/l.day) [5], organic-aqueous two-liquid phase system (0.085 g/l.day) [31], and cloud point systems (1.4 g/l.day) [30] for initial substrate concentrations of 5 g/l (β-sitosterol), 1 g/l (β-sitosterol), and 40 g/l (β-sitosterol:stigmasterol:campesterol, 45:30:25), respectively.

At the end of the fermentation cycle, both the medium and the DFLC were extracted in order to evaluate the overall steroid content in the bioreactor where initial sterol amount was 3 g. Results showed that a total steroid content of about 0.99 g was adsorbed on DFLC fibers with the following relative molar proportions: AD 3.5 %, ADD 8.33 %, β-sitosterol 59.5 %, campesterol 16.6 % and dihydrobrassicasterol 11.9 %. The overall bioconversion rates at the ending were about 57, 76, and 62 % for β-sitosterol, campesterol, and dihydrobrassicasterol, respectively. When experiments were carried out without DFLC, the bioconversion rates of these three sterol species were five times lower than those observed in presence of DFLC (β-sitosterol 12 %, campesterol 14, and dihydrobrassicasterol 12 %).

In this study, AD(D) accumulation was increased fourfold without using chemicals. Moreover, the relative high concentration of AD(D) and low sterol content in aliquots let, together, envisage a green semi-continuous process for AD(D) production.

Conclusions

DFLC can be used in a 5-l jar bioreactor with phytosterols mixture as substrates (1 g/l) and without addition of any chemicals or solubilizing agents. The adequate retention of mycobacterial cells by DFLC increases their interaction with the phytosterol particles and by then the conversion rate. As compared to the control, DFLC increased by four the volumetric productivity of AD(D). Products were accumulated in the aqueous phase while substrates remained on DFLC. This observation lets consider a green semi-continuous process of AD(D) production. DFLC has no influence on cell growth, it is natural, inexpensive, nontoxic, and mechanically strong.

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