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Efficient Bioconversion of High Concentration Phytosterol Microdispersion to 4-Androstene-3,17-Dione (AD) by *Mycobacterium* sp. B3805

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Abstract Low solubility of sterols in aqueous media limits efficient steroid production mediated by biocatalytic microorganisms such as *Mycobacterium*. Sterol emulsion technologies have been developed with low success rates, largely due to the complexity of generating stable and bioavailable particles. In this study, several aqueous dispersions of sterols in-water of different particle sizes were bioconverted to 4-androstene-3,17-dione (AD) in a solvent-free environment, using a classic microorganism *Mycobacterium* sp. B3805 as a model system. According to our results, the high concentration (20 g/L) phytosterol dispersions with the smallest particle size tested (370 nm) achieved up to 54% (7.4 g/L) AD production yield in 11 days. Moreover, the use of 0.1 biomass/sterols ratio in a complex bioconversion media containing yeast extract, and a 1:1 glucose/microdispersion ratio in the presence of the surfactant DK-Ester P-160 (HLB16), allowed homogenization and increased microdispersion stability, thus achieving the best results using emulsion technologies to date.

Keywords Bioconversion · Phytosterols · Microdispersion · AD · Steroids

Introduction

The pharmaceutical steroid industry is mainly focused on the production of two key precursor compound C-17-ketosteroids: 4-androstene-3,17-dione (AD) and 1,4-androstediene-3,17-

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dione (ADD). These precursors comprise the base for the production of highly demanded steroid hormones such as estrogen, progesterone, testosterone, and corticosteroids. The main raw material for the production of AD and ADD through bioconversion with microorganisms is plant-derived sterols (phytosterols) [1–3].

Several studies and technologies have been published in recent years regarding the bioconversion of sterols to produce AD(D). However, in most of them, the initial concentration of sterols reported is less than 5 g/L in batch cultures, causing serious scaling problems [2, 4–6]. This is largely due to its low solubility in aqueous media. The chemical structure of phytosterols is similar to that of cholesterol, resulting in a very low solubility in water and limited solubility in common dietary oils [7]. In the few works with initial phytosterol concentrations of 10 g/L or more, the use of oil/aqueous toxic solvent biphasic systems or expensive compounds is necessary and GMOs are almost essential for efficient yields [3, 8].

A sterol solubility in water of 0.01–0.1% has been determined previously [9, 10]. Therefore, several studies aimed at increasing the concentration of sterols and bioconversion have been conducted, such as surfactant containing sterol emulsions, rational media designs, one or two phases with organic solvents systems, cyclodextrin inclusion complexes, cloud point systems, and the use of adsorbent resins and green solvents such as polymeric liquids, supercritical fluids, ionic liquid, and vegetable oil [3, 11, 12].

The formulation of emulsions and microemulsions [13] to improve the mass transfer interface area to increase the bioavailability of phytosterols has been achieved with modest results [14, 15]. High melting temperatures, low solubility in aqueous phase, and physicochemical instability have led to difficulties in the improvement of this approach of phytosterol fermentation [16]. In addition, the literature suggests that the sterol microemulsion conversion efficiency is affected by the size and shape of the sterol particles formed. Therefore, smaller microemulsions would be a suitable substrate to improve bioavailability, due to its stable structure, thus decreasing the problem of mass transfer to microorganisms [6]. The aforementioned hypothesis has been confirmed by studying the effect of particle sizes in cholesterol emulsions of 10 and 200 μm, achieving greater results of AD production with smaller emulsions [17]. Noh et al., in 2004/US0152153 [18], studied cholesterol emulsion utilization with sucrose ester surfactant. In this patent, comparative assays with the most efficient protocols known in the prior art were performed, reaching 2.6 g/L of AD from cholesterol at 5 g/L, in 5 days of biotransformation, using *Mycobacterium fortuitum* EUG-119.

Although various methods using surfactants have improved the conversion of phytosterols to AD(D), these have been scarce and some of them are industrially unscaled. Therefore, new methods are needed in this area to improve fermentation productivity and efficiency processes to produce AD from phytosterols.

With the aim to improve AD production in an eco-friendly, cost-effective process, using the technology published in the Harting T. et al., 2012/US0046254 patent [19] as a model, which examines the use of microdispersions in the food industry, in this work, we studied the effect of different sized particle with different phytosterol oil-in-water (O/W) microdispersions at relatively high substrate concentrations (> 5 g/L), and the effect of the media and bioconversion conditions on the AD production yields, using a classic microorganisms, such as *Mycobacterium* sp. B3805.

Materials and Methods

Reactives

Mycobacterium sp. B3805 was obtained from the German Resource Centre for Biological Material. Yeast extract and agar were obtained from Difco (USA), glucose p.a. grade. Salts were supplied by Merck (Germany), and AD and Tween 80 were obtained from Sigma (USA). Phytosterols (Phy) from a tall-oil effluent from the paper pulp industry (over 98%, with a β-sitosterol content of 78%) were obtained from Arboris (USA). Sucrose ester tensoactive DK-ester P-160 (HLB 16) was obtained from Dai-ichi Kogyo Seiyaku (Japan). All other chemicals were of analytical or HPLC grade and from various other suppliers.

Phytosterol Dispersion Formulation

Several dispersions were prepared from wood sterols with different particle sizes from the method disclosed in the US patent application of Harting T. et al., 2012/US0046254, with modifications [19]. Dispersions were designated as A10, S15, A25, and A25S and differed in the surfactant percentage used: 0.1% Tween 80 and fatty acids sodium salts from sunflower oil (SSSFA), 0.65% Tween 80, 0.65% oleate sodium and 0.65% sodium oleate plus 0.1% saponin, respectively. In the 1-L Parr reactor, phytosterols, water, and surfactants were charged. The closed reactor is evacuated during 2 min with stirring at 30 rpm. Then, the stirrer speed is increased to 700 rpm and the reactor content heated to 160 °C for 10 min. The former dispersion is fed to an APV Gaulin MR-15 homogenizer and homogenized in one stage at 300 bar. The homogenized mixture is left to cool down to room temperature to form of the final phytosterol microdispersion In the A10 microdispersion, a Non-A10 control was prepared; its microdispersion components were not circulated through the homogenizer machine. The same phytosterols with no surfactant or homogenization process (Phy) were also used as controls. Finally, in order to obtain the Noh emulsion, wood sterols and emulsified sucrose fatty acid esters were heated to melting point, completely dissolved and mixed at 1:0.5 ratio (*w*/*w*), and finally, the mixture was stirred at 80 °C as described in more detail in the protocol claimed in example 2 of the patent of Noh et al., 2004/US0152153 [18].

Mycobacterium sp. B3805 Growing Conditions

The microorganism was cultured at 30 °C in 500-mL Erlenmeyer flasks with a 100-mL culture media (in g/L) (20 glucose; 6 yeast extract; 0.8 K_2HPO_4; 0.6 $MgSO_4{\cdot}7H_2O$; 0.14 $FeSO_4{\cdot}7H_2O$; 5 NH_4NO_3). Then, one fourth volume was transferred to a 1-L flask with 250 mL of culture media with 0.5 g/L of A10 plus 0.8% Tween. Procedures were performed under the same operating conditions for 3 days until the pre-stationary phase of growth was reached. The fermented broth was centrifuged at 3000*g* for 15 min at room temperature; the pellet was subsequently rehydrated at least three times and resuspended in H_2O.

Bioconversion of Phytosterol Dispersions

Bioconversion stoichiometry was defined on the RB parameter basis (biomass/sterols ratio) $RB = \frac{[Biomass] \times \mathrm{Vol}.Biomass}{[Sterols] \times \mathrm{Vol}.Sterols}$ 20. The pellet inoculum was prepared, and the bioconversion was performed in a fermentation media composed of the following (in g/L): 5 glucose, 1.5 yeast extract, 0.31 $MgSO_4x7H_2O$, 0.06 $FeSO_4x7H_2O$, 1.25 NH_4NO_3, 0.01 $CoCl_2x6H_2O$, 0.09 MoO_3, 2.57 DK-Ester P-160 plus 100 mM K_2HPO_4/KH_2PO_4 pH 7 buffer. The bioconversion reactions were conducted at 20 mL in 100-mL flasks and under the following conditions: 0.1 RB, 4-day reaction at 30 ° C. The fermentation media in bioconversion reactions was referenced at 1:1 ratio (phytosterols/glucose), except for the original formula. All experiments were performed with $n \geq 3$, in each figure; average and standard deviation are indicated.

Sterols and AD Quantification

Before the sterol quantitative determination was made, an extraction of AD and phytosterols with ethanol/toluene (3/1) from the fermentation media was performed. Bioconversion was evaluated with GC-FID chromatography using cholesterol with the internal standard technique in the following conditions: carrier: helium 68.4 (64 cm/s) continuous flow; column: HP-5 (5% cross-linked phenyl methyl silicone); injection: split (30:1), 0.5 μL; detector: FID 300 °C temperature 200 °C (10 min), 5 °C/min (20 min), 300 °C (10 min). Bioconversion parameters were defined by the following equations:

$$\mathrm{AD}\ yield\ (\%) = \frac{\mathrm{AD}\ weight}{Initial\ substrate\ weight} \times \frac{Substrate\ molecular\ weight}{\mathrm{AD}\ molecular\ weight} \times 100$$

$$Phytosterol\ conversion\ (\%) = \left(1 - \frac{Final\ substrate\ weight}{Initial\ substrate\ weight}\right) \times 100$$

Particle Size Measurement

To measure the mean particle size, samples were diluted using distilled water in order to score an attenuator index between 5 and 6. Diluted sample were added in a 1-cm cell to carry out the measurement in a Zetasizer Nano S (Malvern, UK) [21].

Results and Discussion

Effect of the Particle Size of Phytosterol Dispersions and Surfactant Addition on Bioconversion Yield

In order to analyze the effect of the particle size in the bioconversion of phytosterols to AD, four 20 g/L phytosterol dispersions of different sizes were generated and incubated for 4 days in the presence of *Mycobacterium* sp. B3805. The effect of surfactant DK-ester P-160 in the fermentation media was evaluated (Fig. 1). The smallest particle size (A10) showed higher yields of AD production (46%) and a volumetric productivity of 1.6 g/L/day with the presence of surfactant in the fermentation media. These effects can be explained by an improved substrate bioavailability. In the case of the larger dispersions, a dependent relationship of the

particle size with the AD production yield is not observed, suggesting that other are mechanisms involved in bioavailability. Different studies with phytosterol emulsions show that smaller particles would be suitable for bioconversion. Nevertheless, sizes achieved by different authors were always larger than 10 μm [6, 17, 18]. The A10 microdispersion (370 nm) is up to 27 times less than those indicated for fermentation of plant sterols, being the smallest used in this field to date. This feature provides advantages over conventional emulsion technology: high stability, improved gravity separation, and increased bioavailability of the phytosterols inside. In particles with a radius smaller than 100 nm, thermodynamic stability is high because the free energy of the emulsion is less than the free energy of the oil/water (O/W) phases separately. In larger sized particles, stability tends to decrease, affecting its structure and reducing sterol mass transfer and bioavailability [16, 22, 23]. Malaviya and Gomes (2008d) developed microemulsions with chloroform as a solvent, achieving β-sitosterol maximum solubility of 8 g/L, a particle size reduction of 10–35 μm from 2.3 g/L β-sitosterol, and an AD production of 0.45 g/L [6]. In this context, our results show an AD production of 6.4 g/L, which is 14 times greater than the above technology.

Besides demonstrating the usefulness of the microdispersion A10 technology, the results demonstrated that fermentation media, and especially DK-ester P-160 surfactant, played a key role in enhancing the properties of different substrates, with an average of 20% of AD production yield and 44% of phytosterols conversion (data not shown), contrasted with 6 and 29%, respectively, in the absence of surfactant. This phenomenon can be explained by the formation of spontaneous emulsions (O/W/W) or (O/W) by a surface tension decrease [16, 23–25]. Surfactants with HLB (hydrophile-lipophile balance) between 8 and 18 are suitable for stabilize O/W emulsions. Consequently, DK-ester P-160 surfactant has an HLB of 16 supporting this phenomenon [23].

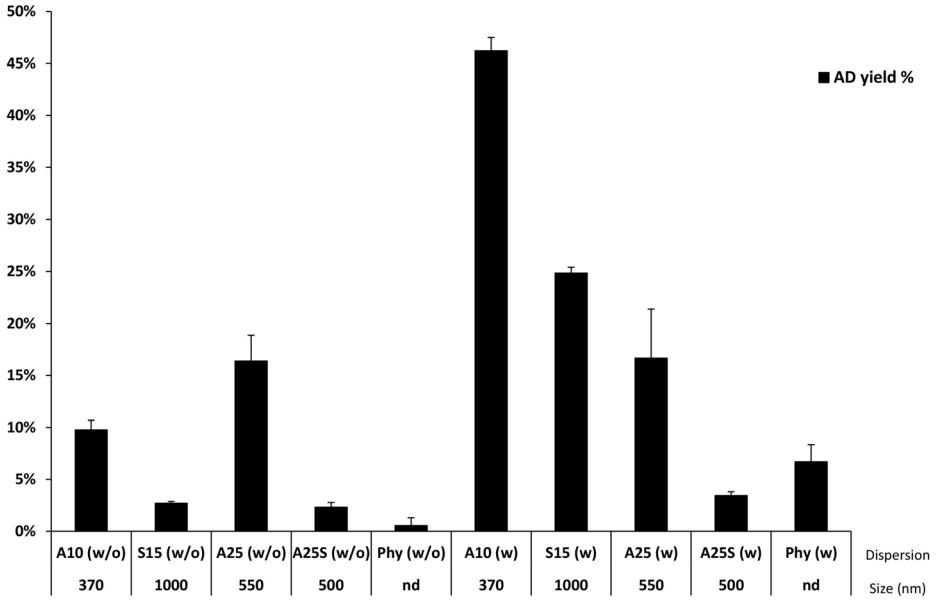

Fig. 1 AD production yield of 20 g/L phytosterol dispersions, at different particle sizes, with (w) or without (w/o) DK-ester P-160 by *Mycobacterium* sp. B3805

Assessment of the Biomass/Phytosterol Ratio (RB)

To optimize AD production levels at different phytosterol concentrations in A10 dispersion with the presence of DK-ester P-160 in the fermentation media, the effect of different biomass/phytosterol relationships (RB) were assessed. Figure 2 shows that at RB 0.1, the increase of substrate load from 5 to 20 g/L does not significantly reduce AD production levels. Increasing the phytosterol concentration in fermentation reactions is difficult due to low solubility and the product inhibition phenomenon [4]. On the other hand, it has been shown that there is a Gaussian distribution for the phytosterol bioconversion efficiency versus the amount of biomass. Thus, it has been suggested that bioconverting enzymes are not produced at low biomass concentrations. In contrast, at high biomass concentration levels, there would be nutrient and oxygen limitations, as would be observed at RB 0.4 and consequently would show a strong decrease in AD production [3, 26]. Finally, RB 0.1 of *Mycobacterium* sp. B3805 is equivalent to the inoculum size of 10–20% reported previously for other bacteria [3, 20, 26–28].

Due to the presence of sterol flocks at RB > 0.1, the effect of yeast extract in the fermentation media on bioconversion of A10 was assayed. In the absence of yeast extract, flocculation in A10 disappears, increasing the AD production at RB 0.2. This suggests the presence of yeast increase viscosity, and thus would trigger the decrease in mass and oxygen transfer in the fermentation reaction, limiting the system for the AD production (data not shown). On the other hand, at RB 0.1, the presence of yeast extract produced a proper condition for the components of the fermentation media and biomass as an appropriate balance of the microdispersion within an environment that promotes bioconversion (data not shown). Marques et al. obtained higher AD production using yeast extract containing media at RB 0.1, rather than in a defined media, from β-sitoesterol as a substrate and glycerol as a carbon source, in the presence of *Mycobacterium* sp. NRRL B-3805 [29]. Consequently, we determine our optimal RB at 0.1.

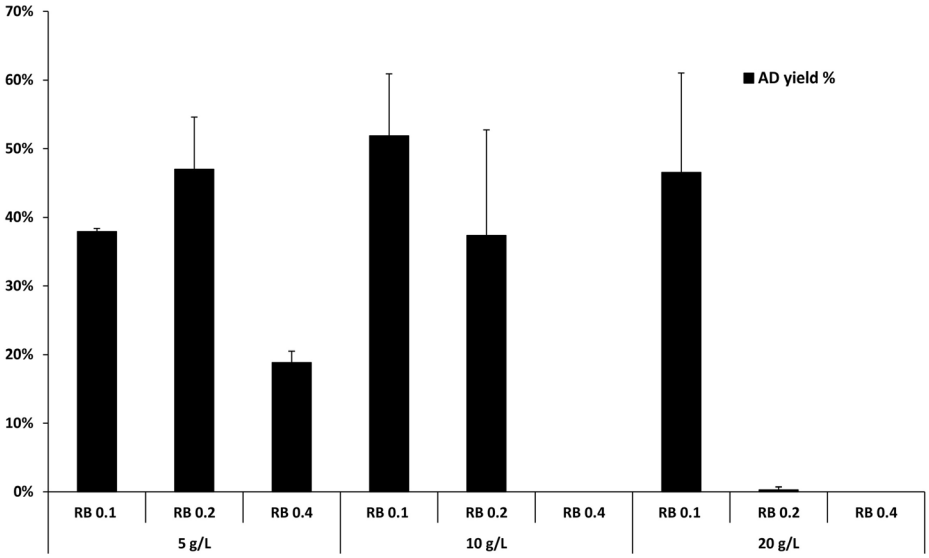

Fig. 2 AD production yield of A10 microdispersion at different biomass/phytosterol ratio (RB) by *Mycobacterium* sp. B3805

Effect of the Fermentation Media, Phytosterol Concentration, and Dispersion Technologies on Bioconversion Yield

To evaluate the effect of phytosterol dilutions on the bioconversion parameters, an A10 microdispersion was assayed in the optimal bioconversion conditions determined above (presence of surfactant, yeast extract and RB 0.1), in a phytosterol/glucose ratio of 1:1. At the same time, incubations under the same conditions with the emulsion technology from Noh S. et al., 2004/US0152153 [18] were performed in order to compare the influence of particle size and to discard the effect of the microdispersion composition. Moreover, incubations with A10, not homogenized (Non-A10), were used as controls.

Figure 3 shows that, with all phytosterol concentrations tested, A10 has a higher yield of AD production than what is shown in other technologies, particularly the Noh emulsion. These results can be achieved through the use of a better mass transfer due to particle size (370 nm vs 10 μm). However, Non-A10 and Phy samples produce similar and even higher levels of AD than Noh, indicating that the particle size in these cases is not the only cause of high AD production levels, and the fermentation media might be involved as a mediator for the generation of spontaneous emulsions, more stable and efficient than the Noh technology. Noh S. et al. (2004) generated cholesterol emulsions with sucrose esters and studied phytosterols use but did not perform experiments with the technology used in our study, which could explain these results. Finally, when using increasing concentrations of phytosterols, the bioconversion parameters decreased for A10, Non-A10, and Noh, as expected. This has been ascribed due to the saturation of the system with substrate, product inhibition, decreased mass, and oxygen transfer promoted by high viscosity or emulsion instability given by interfacial and physicochemical phenomena unique to emulsions [4, 22, 30–32]. Due to the flasks and the volumes handled in this work, it is difficult to distinguish physicochemical phenomena, except

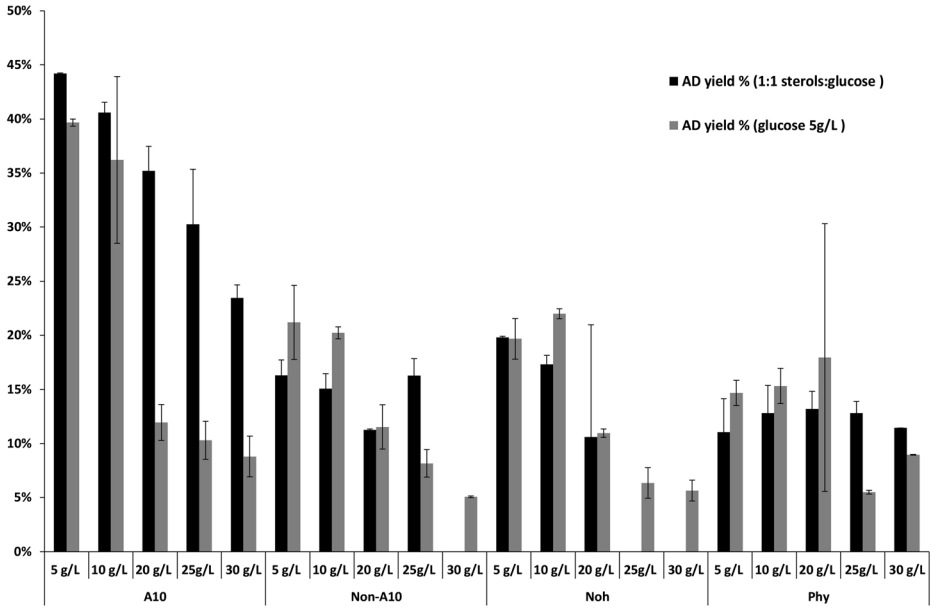

Fig. 3 AD production yield of phytosterol dispersions at different concentrations, using different technologies in 1:1 sterols/glucose standard media and fixed glucose concentration (5 g/L) by *Mycobacterium* sp. B3805

for phytosterols at concentrations higher than 20 g/L. The velocities at which these degradation processes occur are considerably different and vary depending on the size and composition of the fermentation media as well as the operational conditions to which the emulsion are exposed, which is better distinguished in reactors but not in flasks (data not shown) [25, 30].

To determine the effect of the fermentation media on bioconversion parameters, the same experiments were performed with a fixed glucose concentration of 5 g/L (Fig. 3**)**. At the phytosterol concentrations of 5 and 10 g/L, A10 showed AD production yields of around 40%, duplicating the other formulations assayed. In particular, in all concentrations tested, A10 was better than the Noh technology. Nevertheless, at concentrations higher than 10 g/L, all of the approaches that were tested performed similarly in the AD bioconversion parameter. Phy showed comparable levels of AD as A10, which suggests that the media solubilized the phytosterols, producing spontaneous emulsions promoted by the surfactant-contained media, allowing the interaction between the substrate with the microorganism and its enzymes [24, 33]. Hence, the small size of the A10 microemulsion (370 nm) provided an advantage over conventional emulsions such as Noh technology (10 μm) and the nonhomogenized substrates (Non-A10 and Phy) in the AD production yield. Additionally, the fermentation media dilution affected the A10 performance [16]. Thus, higher concentrations of media (Fig. 3) not only improved bioavailability and some physicochemical aspects of A10 but may also allow *Mycobacterium* to exit from a resting state, producing higher levels of active biomass and increasing metabolic flows to produce AD.

Time Course of A10 Bioconversion

A time course of bioconversion of 20 g/L phytosterols in A10, with RB 0.1 plus DK-ester P-160 surfactant and 1:1 phytosterol/glucose ratio of fermentation media (Fig. 4), showed a

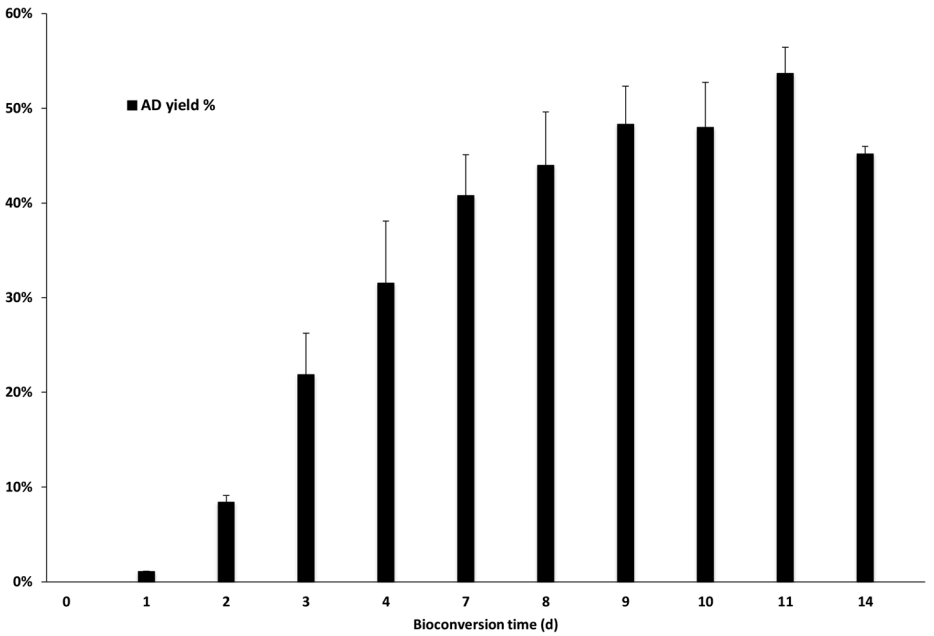

Fig. 4 Time course of AD yields of A10 microdispersion at 20 g/L phytosterols by *Mycobacterium* sp. B3805

gradual increase of AD production yields, reaching 54% (7.4 g/L) on day 11, and a slightly decreasing to 45% (6.4 g/L) on day 14. However, values reached at day 9 did not presented substantial variations on the following days. This was likely due to the lacking of 9α-hydroxylase (KSH) and 3 keto-steroid isomerase (KSTD) enzymes in this Mycobacterium strain [34, 35]. However, the gene cluster kstR2 and the cyclopentanoperhydrophenanthrene (CPF) ring degradation genes, such as fadD3, could have been induced by the presence of phytosterols, such as small amounts of the CPF degradation metabolite 3α-H-4α (3′-propanoate)-7β-methylhexahydro-1,5-indanedione (HIP) [36]. Meanwhile, it has been shown that AD(D) inhibits cell growth and viability, thereby suppressing the sterols transforming activity of *Mycobacterium* [37]. In this regard, the AD(D) removal by XAD-7 resins from *Mycobacterium* sp. B3805 cultures promotes sterol side-chain degradation [38]. Furthermore, the inactivation of *Mycobacterium* sp. NRRL B-3683 was evidenced by the addition of small quantities of exogenous ADD, since only 3.6% of cells remain viable after the addition of 1 g/L ADD [39]. In other organisms such as *Saccharomyces cerevisiae*, the inhibition of the cellular respiration, glucose uptake and growth by AD(D) has been studied [40]. The maintenance as well as the decrease of these specified bioconversion parameters could be an inherent process of the dispersion. It is known that most emulsions become unstable over time due to several physicochemical instability phenomena. The yield of the degradation processes is considerably different and varies, depending on factors such as the size, composition of the fermentation media, and operational conditions to which the emulsions are exposed [25].

The specific consumption of the different phytosterols present in A10 was analyzed in the same time course. 75% of the phytosterols were consumed on day 14, but no clear preference

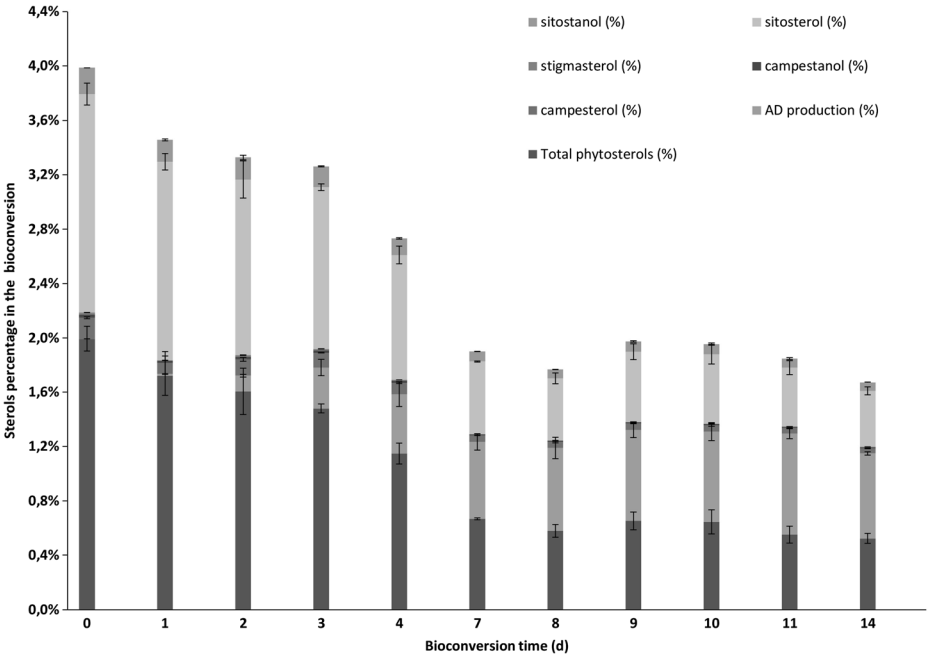

Fig. 5 Time course of specific consumption of the different phytosterols present in A10 microdispersion (20 g/L) and AD production yield, expressed as percentages of the fermentation media

for a particular phytosterol was observed (Fig. 5). The β-sitosterol (the most abundant in A10) was the second most efficiently consumed, along with campesterol and campestanol, which remained at 25.6, 24.6, and 28.1%, respectively, on day 14. Stigmasterol and sitostanol, with a 34.7 and 32.3% of remaining substrate were the least consumed or least efficiently metabolized. Sripalakit et al. compared the consumption of sitosterol, cholesterol, stigmasterol, and ergosterol independently at 0.2 g/L, with the *Mycobacterium* sp. DSMZ2966 and DSMZ2967 strains. The β-sitosterol (the most apolar) was the best substrate for bioconversion, with 63.2% converted to AD on day 14, analogous to our results, while conversion to other substrates such as cholesterol, stigmasterol, and ergosterol were 31.2, 28.8, and 32.2%, respectively [37]. Thus, it can be stated that preference will be given to substrate because of a structural feature of the substrate rather than for its solubility or absolute amounts.

In order to understand the results achieved, Table 1 compares the results of high phytosterol concentration with different advanced technologies. Using the technology developed in this work, it is possible to produce up to 7.4 g/L AD in 11 days of fermentation. Analyzed in terms of volumetric productivity, it is possible to produce 1.2 g/L/day AD (4.8 g/L AD in 4 days) (Fig. 3), a value considerably superior to the other technologies. Zhang et al. used emulsified phytosterols at 10 and 15 g/L with hydroxypropyl-β-ciclodextrynes (1:1–3) with *Mycobacterium neoaurum ZJUVN-08,* bioconverted 6.0 and 2.7 g/L AD in 5 days (1.2 and 0.54 g/L/day) [43]. On the other hand, Kutney et al. reported 80% of AD conversion in 25 days from 30 g/L emulsified phytosterols with polypropyleneglycol using *Mycobacterium* sp. MB 3683, which corresponds to 0.66 g/L/day [42]. Yuan et al. reported a volumetric productivity of 0.44 g/L/d

Table 1 Comparatives results from the state of the art

Sterols concentration (g/L)	Technology	Microorganism	Volumetric productivity (g/L/day)
5	Emulsification with sucrose fatty acid ester [18]	*Mycobacterium fortuitum* EUG-119 (KCCM-10259)	0.51
70	Inclusion complexes with HP-β-CD [8]	*M. neoaurum* NwIB-R10*choM2*	5.8
10	Inclusion complexes with HP-β-CD [8]	*M. neoaurum* ZJUVN-08	1.20
15	Inclusion complexes with HP-β-CD [41]	*M. neoaurum* ZJUVN-08	0.54
10	Sunflower oil/aqueous biphasic systems [3]	*Mycobacterium* sp. MB 3683 5,92	1.18
15	Emulsification with Tween 80 [21]	*Mycobacterium neoarum* NwIB-R10	1.14
10	Solubilizing agent polypropyleneglycol (PPG) [42]	*Mycobacterium* sp. MB 3683	0.52
30	Solubilizing agent polypropyleneglycol (PPG) [42]	*Mycobacterium* sp. MB 3683	0.66
5	Homogenization with Tween 80 and SSSFA (this work)	*Mycobacterium* sp. B3805	0.38
10	Homogenization with Tween 80 and SSSFA (this work)	*Mycobacterium* sp. B3805	0.7
20	Homogenization with Tween 80 and SSSFA (this work)	*Mycobacterium* sp. B3805	1.21
25	Homogenization with Tween 80 and SSSFA (this work)	*Mycobacterium* sp. B3805	1.3
30	Homogenization with Tween 80 and SSSFA (this work)	*Mycobacterium* sp. B3805	1.21

using a solvent free technology [26]. Gao et al. developed an efficient resting cell phytosterol bioconversion process with a genetically modified microorganism strain (*Mycobacterium neoaurum* NwIB-yV) in the presence of hydroxypropyl-β-cyclodextrin (HP-β-CD); however, the authors mentioned that HP-β-CD is a relatively expensive compound and more validations would be needed in order to scale the process [8]. Yao et al. reported 6.85 g/L AD with the genetically modified strain *Mycobacterium neoarum* NwIB-R10 and 4.53 g/L AD using the WT organism strain from 15 g/L emulsified phytosterols in Tween 80 in 6 days of fermentation [44]. Thus, the inclusion of specific genetically modified microorganisms in our technology could highly improve AD production yields. Finally, from 10 g/L phytosterols and sunflower oil as a co-solvent or second phase, we found 85% AD yields and 23% AD yields in a one-phase control, suggesting the usefulness of co-solvents in steroids bioconversion [41].

Conclusion

This study shows that solvent-free phytosterol microdispersions at 20 g/L and the presence of DK-Ester P160 surfactant, in a fermentation media at 1:1 phytosterol/glucose ratio, is an efficient and eco-friendly alternative technology that can be used to improve the bioconversion yields of phytosterols to AD. However, in enhanced AD production conditions, *Mycobacterium* sp. B3805 does not show a clear preference for a particular sterol. Our data suggests the need of further studies to scale this technology and to enhance efficiency by using modified microorganisms and/or solvent addition.

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Compliance with Ethical Standards

Disclosures The authors declare that they have no conflicts of interest.

References

1. Rodina, N. V., Molchanova, M. A., Voishvillo, N. E., Andryushina, V. A., & Stytsenko, T. S. (2008). Conversion of Phytosterols into Androstenedione by Mycobacterium neoaurum. *Applied Biochemistry and Microbiology, 44*, 56–62.
2. Wang, F., Yao, K., & Wei, D.. (2011). From Soybean Phytosterols to Steroid Hormones. In Agricultural and Biological Sciences (Prof. Hany El-Shemy Ed.), ISBN 978-953-307-535-8, InTech. pp 231-252.
3. YG, X., Guan, Y. X., Wang, H. Q., & Yao, S. J. (2014). Microbial side-chain cleavage of phytosterols by Mycobacteria in vegetable oil/aqueous two-phase system. *Applied Biochemistry and Biotechnology, 174*, 522–533.
4. Donova, M. V., & Egorova, O. V. (2012). Microbial steroid transformations: current state and prospects. *Applied Microbiology and Biotechnology, 94*, 1423–1447.
5. Donova, M. V., Nikolayeva, V. M., Dovbnya, D. V., Gulevskaya, S., & Suzina, N. E. (2007). Methyl-beta-cyclodextrin alters growth, activity and cell envelope features of sterol-transforming mycobacteria. *Microbiology, 153*, 1981–1992.

6. Malaviya, A., & Gomes, J. (2008). Nutrient broth/PEG200/TritonX114/Tween80/Chloroform microemulsion as a reservoir of solubilized sitosterol for biotransformation to androstenedione. *Journal of Industrial Microbiology & Biotechnology, 35*, 1435–1440.
7. Rossi, L., Seijen, T., Jack, W. M., Melnikov, S. M., & Velikov, K. Colloidal phytosterols: synthesis, characterization and bioaccessibility. *Soft Matter, 6*(5), 928.
8. Gao, X. Q., Feng, J. X., Wang, X. D., & Hua, Q. (2016). Enhanced Steroid Metabolites Production by Resting Cell Phytosterol Bioconversion. *Chemical and Biochemical Engineering Quarterly, 29*, 567–573.
9. Stefanov, S., Yankov, D., & Beschkov, V. (2006). Biotransformation of Phytosterols to Androstenedione in Two Phase Water-oil Systems. *Chemical and Biochemical Engineering Quarterly, 20*, 421–427.
10. Wang, Z. (2007). The potential of cloud point system as a novel two-phase partitioning system for biotransformation. *Applied Microbiology and Biotechnology, 75*, 1–10.
11. Cabral, J., Fernandes, P., Marques, M., & Carvalho, F. (2009). Screening for suitable solvents as substrate carriers for the microbial side-chain cleavage of sitosterol using microtitre plates. *Process Biochemistry, 44*, 556–561.
12. Shao, M., Zhang, X., Rao, Z., Xu, M., Yang, T., Li, H., & Xu, Z. (2015). Enhanced Production of Androst-1 , 4-Diene- 3 , 17-Dione by Mycobacterium neoaurum JC- 12 Using Three-Stage Fermentation Strategy. *PLoS One, 10*, 1–13.
13. Wang, Z., Neves, M., Isoda, H., & Nakajima, M. (2015). Preparation and Characterization of Micro/Nano-emulsions Containing Functional Food Components Japan. *Journal of Food Engineering, 16*(4), 263–276.
14. Engel, R., & Schubert, H. (2005). Formulation of phytosterols in emulsions for increased dose response in functional foods. *Innovative Food Science & Emerging Technologies, 6*, 233–237.
15. Leong, W. F., Lai, O. M., Long, K., Che Man, Y. B., Misran, M., & Tan, C. P. (2011). Preparation and characterisation of water-soluble phytosterol nanodispersions. *Food Chemistry, 129*, 77–83.
16. Mcclements, D. J. (2012). Crystals and crystallization in oil-in-water emulsions : Implications for emulsion-based delivery systems. *Advances in Colloid and Interface Science, 174*, 1–30.
17. Voishvillo, N. E., Andryushina, V. A., Savinova, T. S., & Stytsenko, T. S. (2004). Conversion of Androstenedione and Androstadienedione by Sterol-Degrading Bacteria. *Applied Biochemistry and Microbiology, 40*, 463–469.
18. Noh, S. K., Kim, M. K., Yoon, W. T., Park, K. M., & Park, S. O. (2004). Method for preparation of Androst-4-ene-3,17-dione and Androsta-1,4-ene-3,17-dione, US Patent 0152153 A1.
19. Harting, T., & Fuenzalida, M. (2012). Dispersion of phytosterols. US Patent 20120046254 A1.
20. Olivares, A., & Acevedo, F. (2011). Effect of inoculation strategies, substrate to biomass ratio and nitrogen sources on the bioconversion of wood sterols by Mycobacterium sp. *World Journal of Microbiology and Biotechnology, 27*, 2513–2520.
21. Rossi, L., Seijen ten Hoorn, J. W. M., Melnikov, S. M., & Velikov, K. P. (2010). Colloidal phytosterols: synthesis, characterization and bioaccessibility. *Soft Matter, 6*(5), 928–936.
22. Mcclements, D. J., Decker, E. A., & Weiss, J. (2007). Emulsion-Based Delivery Systems for Lipophilic Bioactive Components. *Journal of Food Science, 72*, 109–124.
23. McClements, D. J., & Food Emulsions Principles, Practices, and Techniques. (2004). Implications for emulsion-based delivery systems. *Advances in Colloid and Interface Science, 174*, 1–30 (2012).
24. Sagalowicz, L., & Leser, M. E. (2010). Current Opinion in Colloid & Interface Science Delivery systems for liquid food products. *Current Opinion in Colloid & Interface Science, 15*, 61–72.
25. Mcclements, D. J., & Rao, J. (2011). Food-Grade Nanoemulsions : Formulation , Fabrication, Properties, Performance, Biological Fate, and Potential Toxicity. *Critical Reviews in Food Science and Nutrition, 4*, 37–41.
26. Yuan, J. J., Guan, Y. X., Wang, Y. T., Wang, H. Q., & Yao, S. J. (2016). Side-chain cleavage of phytosterols by Mycobacterium sp. MB 3683 in a biphasic ionic liquid/aqueous system. *Journal of Chemical Technology and Biotechnology, 91*, 2631–2637.
27. Kutney, J. P., Milanova, R., Vassilev, C., Stefanov, S., & Nedelcheva, N. (2000). Process for the microbial conversion of phytosterols to androstenedione and androstadienedione. US Patent 006071714 A
28. Marques, M. P., Carvalho, C., De Cabral, J. M., & Fernandes, P. (2010). Scaling-Up of Complex Whole-Cell Bioconversions in Conventional and Non-Conventional Media. *Biotechnology and Bioengineering, 106*, 619–626.
29. Marques, M., Carvalho, F., Carvalho, C., Cabral, J., & Fernandes, P. (2010). Steroid bioconversion : Towards green processes. *Food and Bioproducts Processing, 88*, 12–20.
30. Bhatti, H. N., & Khera, R. A. (2012). Biological transformations of steroidal compounds: A review. *Steroids, 12*, 1–24.
31. Malaviya, A., & Gomes, J. (2008). Androstenedione production by biotransformation of phytosterols. *Bioresource Technology, 99*, 6725–6737.

32. Mcclements, D. J. (2012). Advances in fabrication of emulsions with enhanced functionality using structural design principles. *Current Opinion in Colloid & Interface Science, 17*, 235–245.
33. Donova, M. V., & Nikolaeva, V. (2005). M and Egorova OV, Enzymes involved in modification of the steroid nucleus of industrial mycobacterial strains: isolation, functions, and properties. *Applied Biochemistry and Microbiology, 41*, 514–520.
34. Yuan, J., Chen, G., Cheng, S., Ge, F., Qiong, W., Li, W., & Li, J. (2015). Accumulation of 9α-hydroxy-4-androstene-3,17-dione by co-expressing kshA and kshB encoding component of 3-ketosteroid-9α-hydroxylase in Mycobacterium sp. NRRL B-3805. *Sheng Wu Gong Cheng Xue Bao, 31*(4), 523–533.
35. Rodríguez-García, A., Fernández-Alegre, E., Morales, A., Sola-Landa, A., Lorraine, J., Macdonald, S., Dovbnya, D., Smith, M. C. M., Donova, M., & Barreiro, C. (2016). Complete genome sequence of 'Mycobacterium neoaurum' NRRL B-3805, an androstenedione (AD) producer for industrial biotransformation of sterols. *Journal of Biotechnology, 224*, 64–65.
36. Casabon, I., Crowe, A. M., Liu, J., & Eltis, L. D. (2013). FadD3 is an acyl-CoA synthetase that initiates catabolism of cholesterol rings C and D in actinobacteria. *Molecular Microbiology, 87*, 269–283.
37. Sripalakit, P., Wichai, U., & Saraphanchotiwitthaya, A. (2006). Biotransformation of various natural sterols to androstenones by Mycobacterium sp. and some steroid-converting microbial strains. *Journal of Molecular Catalysis B: Enzymatic, 41*, 49–54.
38. Huang, C. L., Chen, Y. R., & Liu, W. H. (2006). Production of androstenones from phytosterol by mutants of Mycobacterium sp. *Enzyme and Microbial Technology, 39*, 296–300.
39. Perez, C., Falero, A., Llanes, N., Hung, B. R., Hervé, M. E., Palmero, A., & Martí, E. (2003). Resistance to androstanes as an approach for androstandienedione yield enhancement in industrial mycobacteria. *Journal of Industrial Microbiology & Biotechnology, 30*, 623–626.
40. Donova, M. V., Gulevskaya, S., Dovbnya, D., & Puntus, I. F. (2005). Mycobacterium sp. mutant strain producing 9alpha-hydroxyandrostenedione from sitosterol. *Applied Microbiology and Biotechnology, 67*, 671–678.
41. YG, X., Guan, Y. X., Wang, H. Q., & Yao, S. J. (2014). Microbial side-chain cleavage of phytosterols by mycobacteria in vegetable oil/aqueous two-phase system. *Applied Biochemistry and Biotechnology, 174*, 522–533.
42. Kutney, J. (2003). Process for fermentation of phytosterols to androstadienedione, EU Patent 1 507 867 B1.
43. Zhang, X. Y., Peng, Y., Su, Z. R., Chen, Q. H., Ruan, H., & He, G. Q. (2013). Optimization of biotransformation from phytosterol to androstenedione by a mutant Mycobacterium neoaurum ZJUVN-08. *Journal of Zhejiang University Science B - Biomedicine & Biotechnology, 14*, 132–143.
44. Yao, K., Wang, F., Zhang, H. C., & Wei, D. Z. (2013). Identification and engineering of cholesterol oxidases involved in the initial step of sterols catabolism in Mycobacterium neoaurum. *Metabolic Engineering, 15*, 75–87.