Biotransformation of Phytosterols to Androstenedione in Two Phase Water-oil Systems

S. Stefanov, D. Yankov*, and V. Beschkov*+
Balkanpharma –AD, Razgrad, Bulgaria
*Institute of Chemical Engineering, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Original scientific paper
Received: January 30, 2006
Accepted: September 1, 2006

The microbial transformation of phytosterols to androstenedione (androst-4-ene--3,17-dione, AD) and androstadienedione (androsta-1,4-diene-3,17-dione, ADD) in two-phase water-oil systems by means of the strain *Mycobacterium* sp. *MB 3683* has been studied. The effect of some process conditions, like the agitation speed, the age and amount of inoculum, the temperature and some additional carbon sources have been investigated. The highest conversion rates were attained with 10–15 % of inolucum of age t = 16–20 h at T = 34–35 °C and n = 400 min^{-1}. Media containing high concentrations of carbohydrates have a negative impact on the process, while the natural nitrogen sources influence beneficially the bioconversion. The use of silicon oil and polypropylene-glycol as solubilizing agents was found suitable for the above-mentioned biotransformation and permitted to increase considerably the amount of the substrate in the system. On the other hand, the biotransformation rate depended on the amount of the oils, because they inhibited the microbial cells growth.

Key words:
Phytosterol, androst-4-ene-3, 17-dione, *Mycobacterium*, two-phase water-oil systems

Introduction

The production of many new medicals is associated with the processes of microbial biotransformation of steroids and sterols. Three of the microbial modifications of steroids are of particular interest for the pharmaceutical and chemical industry: the selective cleavage of the hydrocarbon chain, the Δ^1 – dehydrogenation and 11-hydroxylation. Although, many of these bioconversions are well-established, the research efforts are ongoing in order to increase the efficiency of the existing processes as well as to identify new potentially useful bioconversions. Some of the recent developments are summarized in the excellent reviews of *Mahato* and *Garai*,[1] *Fernandes* et al.[2] and *Donova* et al.[3]

The reaction of the side- chain cleavage in the sterols molecules is of a practical interest, since the majority of the pharmaceutically active steroids come from intermediates produced from natural raw materials.

Androstenedione (androst-4-ene-3, 17-dione, AD) is a base of the synthesis of important pharmaceuticals. One of the methods for its commercial production is the transformation (the selective degradation of the side hydrocarbon chain) of cholesterol (of animal origin) or plant-originated β-sterols by mutants of bacterial strains.[4,5] Recently, some papers discussing the possibility of bioconversion of sterol-rich fractions from different industrial wastes have been published.[6–8]

There are two main problems for the industrial production of androstenedione by microbial transformation. The first one is based on the capacity of the used strain to transform the sterols specifically. In this case it is important, that during the process, side products with a similar structure are not accumulated and the main skeleton of the steroid molecule to be retained. Some mutants with appropriate enzyme systems for these biotransformations are isolated.[9–11]

The second problem is related to the concentrations of the sterols, subjected to biotransformation. As a rule, steroids have very low solubility in water, i.e. 0.01 to 0.1 %. Therefore different solvents, mostly alcohols, esters, hydrocarbons,[12–16] for increasing the sterols concentration in the system are used. However, the toxicity of these solvents restricts their use at high concentrations. The application of water-miscible organic solvents, particularly tri-, di- and mono-alcohols has the advantage that in these cases they serve as a carbon source for the microbial cells.[17] *Cruz* et al. have investigated the influence of different esters of mono- and di- carboxylic acids[18] and especially some phtalates.[19]

Cyclodextrins are widely used for introduction of higher concentrations of steroids in the nutrient

+Correspondence: Professor V. Beschkov, Institute of Chemical Engineering, Bulgarian Academy of Sciences, Acad. G. Bontchev str., Bl. 103, 1113 Sofia, Bulgaria; E-mail: bioreac@bas.bg

media.[20–21] Another approach to overcome the problems with solvent toxicity is to use immobilized cells for biotransformations in organic media.[12, 22–26]

Phase and *Patil*[27] have reported that some natural oils are better solubilizing agents for sterols conversion than organic solvents. However, above certain sterol concentration, inhibition of the biotransformation has been observed.

The use of *Mycobacterium sp*. MB 3683 for the microbial biotransformation of androstenedione (androst-4-ene-3, 17-dione, AD) and androstadienedione (androsta-1, 4-diene-3, 17-dione, ADD) was previously described.[28] Moreover, it was shown that the use of appropriate solubilizing agents, like silicon oils and polypropylene-glycols (PPG), can increase the sterols concentration in the nutrient media.

The dissolution of phytosterols in polymer oils and their addition to the main nutrient media appeared to be a suitable option for the biotransformation by the above mentioned strain. The goal of present paper is to study the effect of vegetable and silicon oils, as well as polypropylene glycol oil on the phytosterol to AD and ADD biotransformation.

Materials and methods

Microorganism

The strain used in this study is a mutant of *Mycobacterium sp.* designated as MB 3683 (ATCC number PTA-352).

The strain was maintained on LB medium, containing per litter: bactotrypton – 10; yeast extract -5; NaCl- 10 and agar-agar – 15, all of Difco.

Materials

Silicon oils with different viscosity, known under their commercial name "Silicone fluid-NM", and liquid polypropylene-glycol oil (PPG) "Caradol ED 56–10" (a Shell Chemicals production), were used as solvents. The vegetable oils (sunflower and soybean) were from domestic suppliers (Perseyoil, Razgrad, Bulgaria).

Two different types of phytosterols were used: the first one – Czech production (Pentos) with a fraction (*w*/%) of β-sitosterol, 38.4; campesterol, 32.5; brassicasterol, 25.9; stigmasterol, 0.65, and the second one – product of Cargill Inc, USA , containing mass fraction (*w*/%): β-sitosterol, 44.4; campesterol, 27.5; stigmasterol, 17.9; brassicasterol, 4.2 and sitostanol, 1.1.

Nutrient additives (technical grade) were – corn extract, corn gluten, (both of Amylum, Bulgaria) molasses (Zaharni zavodi PLC, Bulgaria), corn flour, wheat flour (both of Tsarevin, Bulgaria), soya flour, soya protein (both of Sojaprotein, Serbia), fish flour (Copeinca corp., Peru)

Phytosterol dissolution

The phytosterols dissolution was made at 90–140 °C under continuous stirring. Initially the oils were heated to 60 °C, then the phytosterols were added and the heating continue to 90–140 °C, depending on oil type. Then the solution was cooled to 60 °C and an appropriate amount was added to the fermentor. The maximum quantities of dissolved phytosterols for different solubilizing agents are given in Table 1. It is worth to mention, that after the cooling of the vegetable oils with phytosterol mass concentration higher than 100 g l^{-1}, crystallisation of the substrate was observed. The critical mass concentration for the silicone oil was about γ = 50 g l^{-1}, depending on oil viscosity. For PPG such crystallisation was not observed, but at high phytosterol concentration the solutions became very viscous.

Table 1 – *Phytosterol solubility in different oils*

Oil	Phytosterol 1 γ/g l^{-1}	Phytosterol 2 γ/g l^{-1}	Temperature
Sunflower	500	300	90–95 °C
Soybean	600	500	90–95 °C
Silicone	150	100	125–140 °C
PPG	300	200	110–120 °C

Method of cultivation

The inoculum of the strain was prepared in 2 l flasks, containing 500 ml of nutrient medium ***1*** as follows, g l^{-1}: molasses, 54; $NaNO_3$, 5.4; $NH_4H_2PO_4$, 0.6; sunflower oil -20 ml l^{-1} (silicon or PPG oil – 2 ml l^{-1}), ph of the medium 7.0. After reaching biomass concentration about 0.5 g l^{-1} and pH of the medium 7.9–8.0, with the content of the best 6 flasks gathered together a 50 l fermentor, containing 30 l of medium ***1*** was seeded. The reactor was incubated at T = 35 °C , n = 300 min^{-1} and aeration 1 l l^{-1} min^{-1}. Appropriate amount of the culture medium with desired age was transferred to the working reactors.

The biotransformation was carried out in medium ***2*** with the following contents, (g l^{-1}): NH_4NO_3, 2; KH_2PO_4, 1; K_2HPO_4, 2; Na_2HPO_4, 2; KCl, 0.2; $CaCl_2$, 0.3, and as micronutrients (altogether 1 ml l^{-1}): in 1 l water: $ZnSO_4$ –11 g; $MnSO_4$ – 6 g; $FeSO_4$ – 1 g; $CoSO_4$ – 0.3 g; $CuSO_4$ – 0.04 g; H_3PO_3 – 0.03 g; KI – 0.001 g; pH 7.0.

The used salts, carbon and nitrogen sources were of analytical grade from various suppliers.

The nutrient medium was sterilized for 30 min at 120 °C and cooled to 35 °C. At this stage, corresponding oil in appropriate phase ratio (ψ), containing necessary phytosterol concentration (based on total reaction volume) was added. After that, about 3 l of the preliminary prepared inoculum was added to the fermentor, so that the initial biomass concentration was 0.05 g l^{-1}. The total volume was adjusted to 30 l. The volume was maintained constant by addition of sterile water.

The process was carried out in 50 l agitated fermentor at n = 400 min^{-1}, 35 °C and aeration 1 l l^{-1} min^{-1}. The pH of the broth was not controlled during cultivation. In case of vegetable oils, pH decreased from 7.0 to about 5.0, while in case of silicone and PPG the pH value at the end of process was about 6.5.

Analytical methods

Steroids: The analyses were made by gas-chromatograph Pye-Unicam PU 4550 equipped with glass column 1.5 m · 4 mm. The column was packed with 2 % methylphenylsilicon (50 % Ph) on the chromosorb W/AN, DMCS (80–100 mesh). The injection temperature was 295 °C; the flame ionization detector temperature was 300 °C and the oven temperature was 270 °C. Nitrogen (Q = 50 ml min^{-1}) as carrier gas was used.

The samples of 20 ml from the broth were extracted twice with 10 ml dichloromethane.

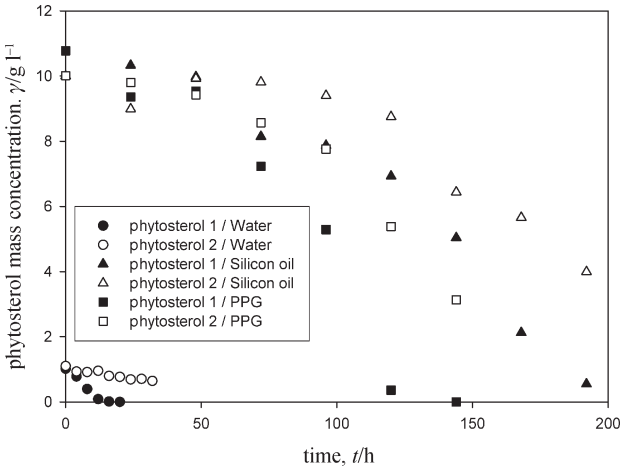
Standards: androstenedione and androstadienedione (both products of Sigma, 98 % purity) were dissolved in dichloromethane. Campesterol 98 %, β-sitosterol 97 %, stigmasterol 95 % and sitostanol-95 % (all of Sigma) were also used.

Cell growth: Twenty milliliters of the broth were extracted with equal amount of chloroform for oil and product removal. After phase separation, the water phase was filtered and twice washed with chloroform and distilled water. The residual cell mass was dried (110 °C) to constant weight.

Results and discussion

The question about the importance of this biotransformation for the microbes' metabolism is very important. It was established that *Mycobacterium MB 3683* degrades specifically only the side hydrocarbon chain in phytosterols molecules and uses it as a carbon source.[28] As a product AD is accumulated in the broth as a major product. Too small amount of ADD, less than 10 % of the total product, was observed as well. In what follows the product concentration is the total sum of the two products. The strain has no potential to degrade the main skeleton of the steroid molecules. If there is no other source of carbon but phytosterols, we can consider the biotransformation as fermentation process with one substrate associated with the microbial growth.

In all experiments the conversion of phytosterol 2 (Cargill Inc., USA) was lower. This is probably due to the different composition of two phytosterols, especially the higher content of sterols with shorter side chain (campesterol and brassicasterol) in phytosterol 1. Some results are presented in Fig 1. The experiments were carried out on medium **2** at 35 °C and n = 400 min^{-1}. As substrate 1 g l^{-1} phytosterol solution in distilled water and 10 g l^{-1} dissolved in silicone or PPG oils (phase volume ratio ψ = 3:7) were used. In what follows only the results obtained with phytosterol 1 (Pentos, Czech Republic) as substrate are presented.

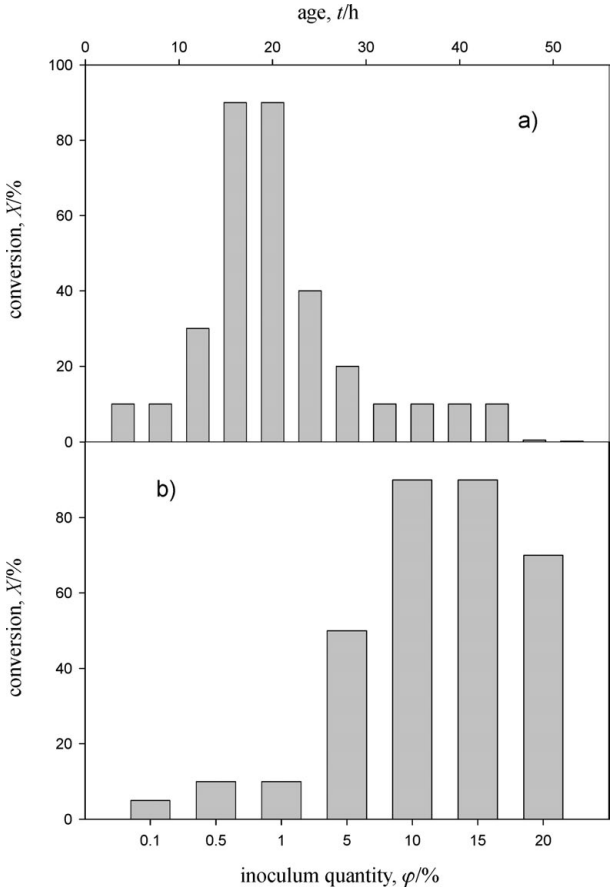


F i g . 1 – *Comparison of phytosterol 1 and 2 degradation with different solubilizing agents – γ = 1 g l^{-1} in distilled water, γ = 10 g l^{-1} in silicon oil and PPG, phases ratio ψ = 3:7*

Influence of the experimental conditions

The effects of the agitation speed, the age and amount of inoculum and of the temperature were studied. In this study the experiments were carried out on medium ***2*** and 10 g l^{-1} phytosterol dissolved in silicon oil. The phase ratio (oil to water) was ψ = 3:7 and the incubation time – 192 h. Some results are shown in Fig. 2 a, b evidencing that very high conversion yields (up to 90 %) are achieved at ψ = 10–15 % of inolucum with an age of 16–20 h.

The cultivation in two-phase system always poses the question of effect the mass transfer on the microbes' development. In the considered case of oil-dissolved steroids these effects become quite significant, because of the high viscosity of the organic phase. It should be noted, that the silicon oil and the polypropylene-glycol are not biodegradable and the phytosterols are the single carbon source.



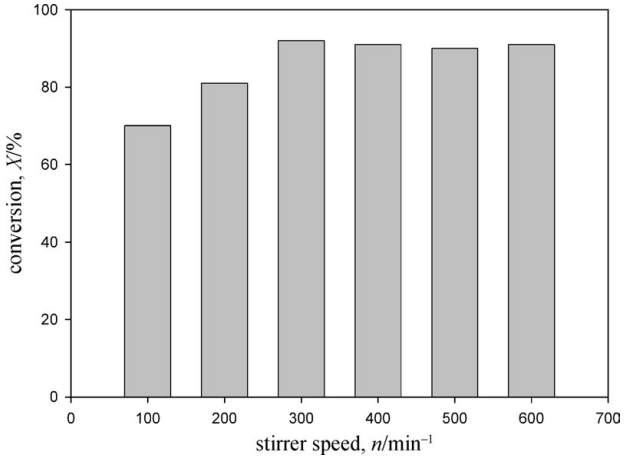
F i g . 2 – *Influence of the age and inoculum quantity (φ/%) on the transformation of phytosterols. Oil to water ratio ψ = 3:7, 10 g l^{-1} phytosterol*

A series of experiments at different agitation speeds were carried out. The results showed that for the adopted experimental conditions (medium ***2***, 10 g l^{-1} phytosterol, silicone oil as solvent, ψ = 3:7 oil to water phase ratio, 192 h of incubation) the biotransformation rate increased on the agitation speed from 100 to 300 min^{-1}. For agitation speeds (n) from 300 to 600 min^{-1} the process rate remained constant. These results are shown in Fig. 3. Similar results were obtained for higher oil to water phase ration, ψ = 6:4, and for polypropylene-glycol as well. Therefore, later experiments were carried out at n = 400 min^{-1}.

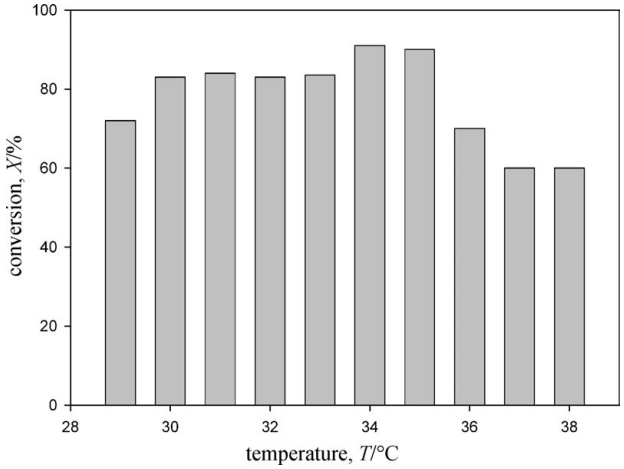
The results for the effect of the temperature (varied within 29 and 38 °C) are shown in Fig. 4. The highest conversion rates were attained at 34–35 °C. The following experiments were carried out at this temperature range.

Effect of nutrient addition

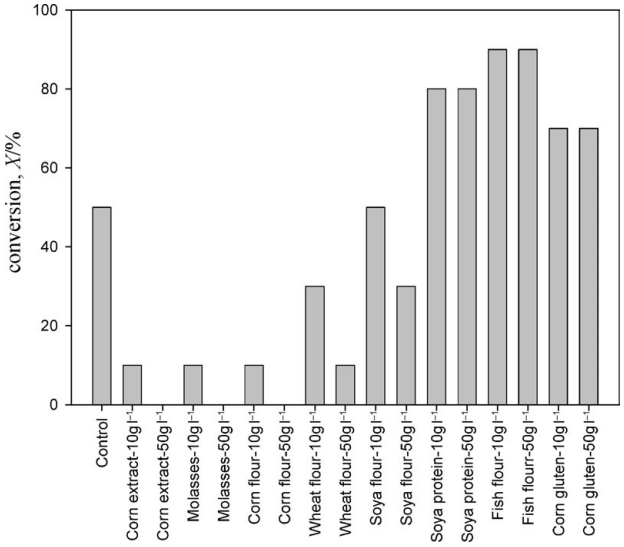
Different nutrient additives to the silicon oil/water (ψ = 3:7) systems (10 g l^{-1} phytosterol) were added. They contained biologically active substances, organic nitrogen compounds, carbon sources and salts. The incubation time was 144 h for each run. The results are shown in Fig. 5.



F i g . 3 – *Influence of the agitation on the phytosterol transformation with silicon oil as solvent. Oil to water ratio ψ = 3:7, 10 g l^{-1} phytosterol*



F i g . 4 – *Effect of the temperature on the phytosterol transformation with silicon oil as solvent. Oil to water ratio ψ =3:7, 10 g l^{-1} phytosterol*



F i g . 5 – *Effect of different media additives on the phytosterol transformation. Silicon oil as solvent, oil to water ratio ψ = 3:7, 10 g l^{-1} phytosterol*

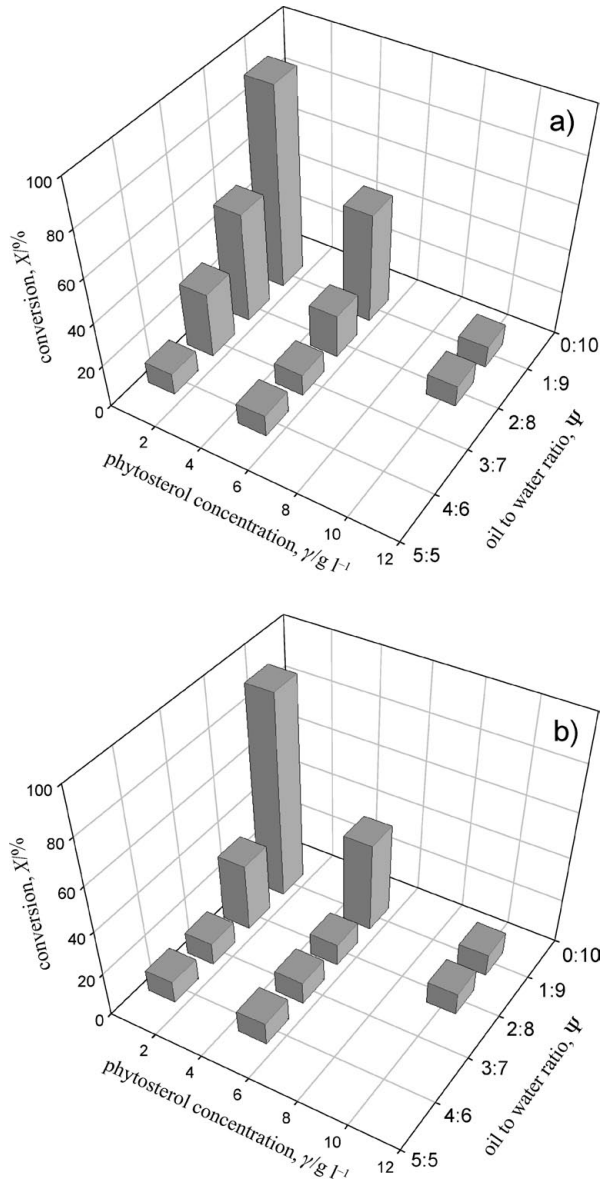
The media containing high concentrations of carbohydrates have negative impact on the biotransformation process, because these types of organic carbon source are preferred by the microorganisms over the sterols. The organic nitrogen compounds influence beneficially the process. Best results are obtained using fish flour in mass concentrations of 10 to 50 g l^{-1}. In these cases the process duration was shortened to 48 h only. None of these additives was used, in the experiments presented below.

Effect of the solubilizing agent

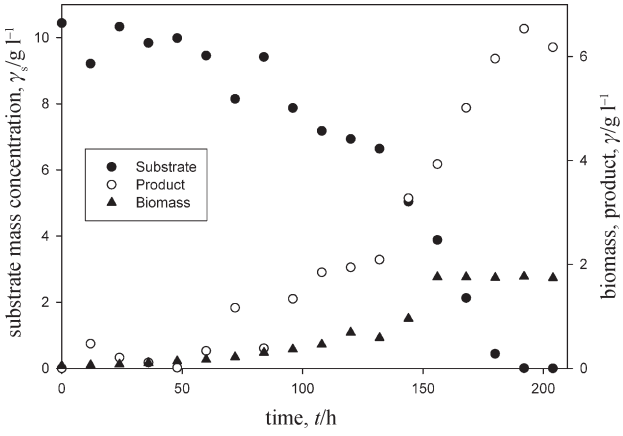
Addition of vegetable oils (like sunflower and soybean ones) enables to increase considerably the phytosterol concentration in the system. As it was mentioned above, it may reach up to 100 g l^{-1}. However, this concentration increase leads to decrease the conversion rate. For example, at initial mass concentration of 1 g l^{-1} (oil to water ratio ψ = 1:9) the conversion may reach 90 %, whereas at 5 g l^{-1} it is 50 %, and at 10 g l^{-1} it is 10 % only, accompanied by substantial extension of the process duration. The origin of the vegetable oil does not affect the process and the conversion rates obtained with phytosterol 2 are lower than that with phytosterol 1. The increase of oil quantity (in order to decrease the viscosity of the phytosterol solution) makes the conversion rate worse additionally. The latter dropped to 50 % at oil to water ratio ψ = 2:8, and to 10 % at ratio ψ = 4:6. The results are shown in Fig. 6a, b. The explanation of this effect are possibly the higher concentrations of fatty acids, being competitive substrate to the sterols for the microbial cells. Similar assumption was suggested by *Donova* et al.[7] What is more, the mechanism of sterol side chain cleavage is similar to this of β-oxidation of fatty acids.[29] The initial sterol concentration, the duration of biotransformation and the concentration of the end product are of particular importance for the perspectives for the industrial application of the studied biotransformation. As usual, downstream processing requires higher conversion degrees and higher androstenedione concentrations.

The transformation of phytosterols to AD and ADD in silicon oil and polypropylene-glycol media was studied for initial concentration of phytosterol 10 g l^{-1} and oil/water ratio ψ = 3:7. The results obtained are shown in Figs. 7 and 8.

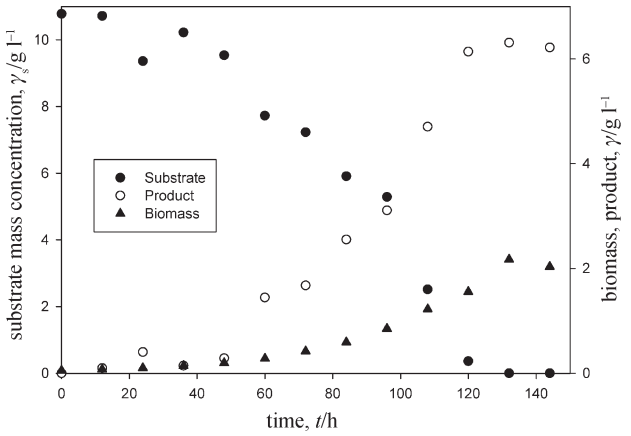
The main difference between two soluibilizing agents is in the duration of the process. When silicone oil is used, the necessary time for nearly complete substrate conversion is about 180 h, while in the case of polypropylene glycol – about 120 h. It is worth to point out that in the latter case the lag-phase is significantly shorter.



F i g . 6 – *Effect of vegetable oils on the phytosterol transformation: a)- sunflower oil; b)-soybean oil*



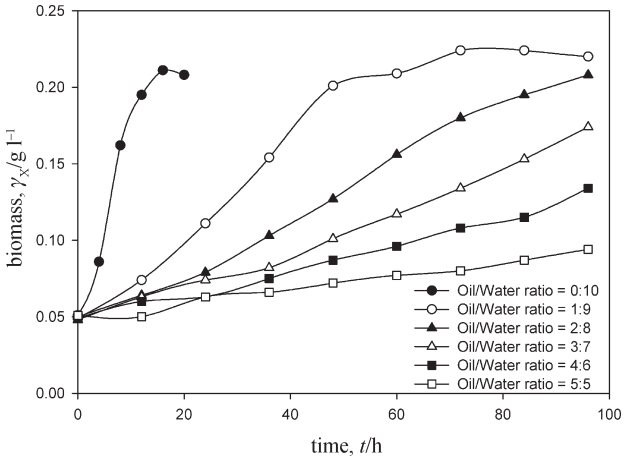
F i g . 7 – *Time course of phytosterol degradation, biomass and product accumulation in case of silicon oil as solubilizing agent. Initial phytosterol concentration -10 g/l, and oil/water ratio 3:7(v/v)*



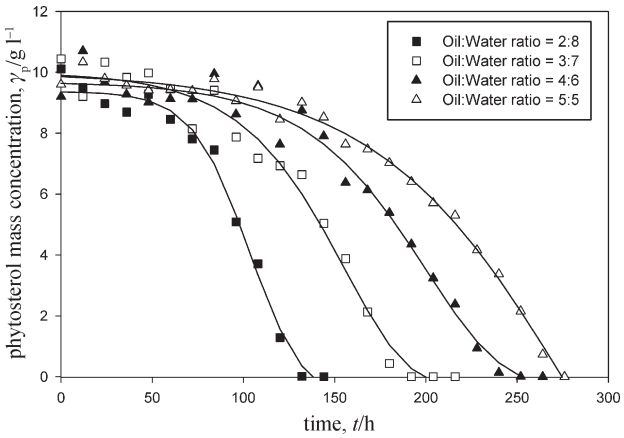
F i g . 8 – *Time course of phytosterol degradation, biomass and product accumulation, in case of polypropilene glycol as solubilizing agent. Initial phytosterol mass concentration* γ = *10 g l*$^{-1}$*, and oil/water ratio* ψ = *3:7*

Since the concentration of phytosterol in the medium is limited by its solubility in oils, one substrate concentration can be achieved by various amounts of different oils. Therefore, experiments at different "silicon oil/water" ratios were carried out. Some results for biomass accumulation during the first 96 h of the process are presented in Fig. 9. In the case of a pure water system phytosterol was preliminary micronized (Airfilco CL-1123 micronizer, 25 μm). When the amount of silicon oil was increased, the biomass accumulation, as well as the biotransformation (results not shown), was delayed. The increase of oil concentration in the nutrient medium leads to increase of the time, necessary for the complete biotransformation. Similar results for polypropylene-glycol-water systems were obtained.

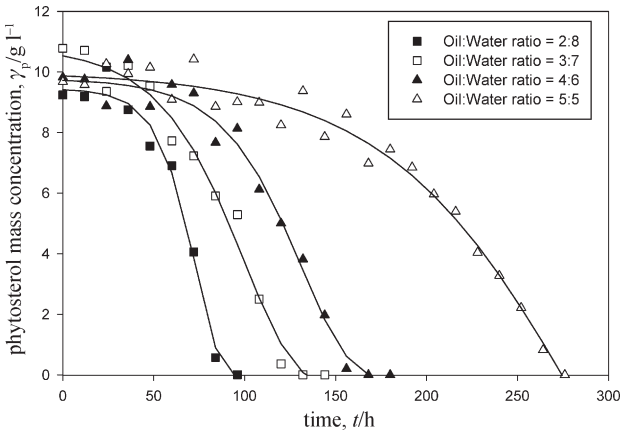
The biotransformation at different phase ratios was studied on medium **2**, but at equal initial sterol mass concentration 10 g l^{-1}. The time profiles for the biotransformation at different phase ratios substrate



F i g . 9 – *Influence of the oil to water ratio on the biomass accumulation. Initial phytosterol mass concentration* γ = *10 g l*$^{-1}$*. Silicon oil as solvent*



F i g . 1 0 – *Influence of the silicon to water ratio on the phytosterol transformation. Initial phytosterol mass concentration* γ = *10 g l*$^{-1}$*, phases ratio* (ψ) *from 2:8 to 5:5*



F i g . 1 1 – *Influence of the polypropylene-glycol to water ratio on the phytosterol transformation. Initial phytosterol mass concentration* γ = *10 g l*$^{-1}$*, phases ratio* (ψ) *from 2:8 to 5:5*

concentration are shown in Figs. 10 and 11. A different impact of the different oils on the biotransformation rate was established. In polypropylene-glycol the transformation rate, up to oil to water ratio ψ = 4:6, was higher. Some further comments and quantitative data on the final product distribution in the system components and location of the cells in the oil-water systems are summarized in the Table 2

Conclusions

The use of silicon oil and polypropylene-glycol as solubilizing agents was suitable for the case of phytosterols to-androstenedione biotransformations. These oils can serve as reservoir for achieving higher initial sterols concentrations in the fermentation system. On the other hand the biotransformation rate depends on the amount of the oils, because they inhibit the microbial cells growth. However, optimization of the nutrient medium and of the oil amount can considerably shorten the complete biotransformation process.

Table 2 – *Some characteristics of the biotransformation process with different oils*

Characteristic	Vegetable oils	Silicon oil	Polypropylene-glycol oil
Phytosterol solubility	Very good	Good	Very good
Toxicity	None	At high concentrations	None
AD degradation	Reduced	Effective but requires prolonged incubation	Reduced at relatively long incubation periods
Where is situated the produced AD?	Mainly in the oil phase, hardly in the cells	Mainly in the cells and water phase, hardly in the oil phase	Mainly in the oil phase and cells, hardly in the water phase

List of symbols

n – stirring speed, min^{-1}
T – temperature, °C
t – age, time, h
Q – volume flow rate
w – mass fraction, %
χ – conversion %
γ – mass concentration, g l^{-1}
φ – volume fraction, %
ψ – volume ratio, V_{oil}/V_{water}

References

1. *Mahato, S. B., Garai, S.,* Steroids **62** (1997) 332.
2. *Fernandes, P., Cruz, A., Angelova, B., Pinheiro, H. M., Cabral, J. M. S.,* Enz. Microb. Technol. **32** (2003) 668.
3. *Donova, M. V., Egorova, O. V., Nikolayeva, V. M.*, Proc. Biochem. **40** (2005) 2253.
4. *Ahmad, S., Roy, P. K., Khan, A. W., Basu, S., Johri, B. N.,* World J. Microb. Biotechnol. **7** (1991) 557.
5. *Liu, W., Lee, C.,* J. Chin. Argic. Chem. Soc. **30** (1992) 52.
6. *Dias, A. C. P., Fernandes, P., Cabral, J. M. S., Pinheiro, H. M.,* Bioresurce Technol. **82** (2002), 253.
7. *Donova, M. V., Dovbnya, D. V., Sukhodolskaya, G. V., Khomutov, S. M , Nikolayeva, V. M., Kwon, I., Han, K.,* J. Chem. Technol. Biotechnol. **80** (2005) 55.
8. *Perez, C., Falero, A., Hung, B. R., Tirado, S., Balcinde, Y.,* J. Ind. Microbiol. Biotechnol. **32** (2005), 83.
9. *Egorova, O., Gulevskaya, S., Puntus, I., Filonov, A., Donova, M.,* J. Chem. Technol. Biotechnol. **77** (2002) 141.
10. *Vidal, M., Bacerra, Y., Mondaca, M., Silva, M.,* Appl. Microbiol. Biotechnol. **57** (2001) 385.
11. *Voishvillo, N. E., Andryshina, V. A., Savinova, T. S., Stytsenko, T. S., Vasileva, N. A., Turova, T. P., Kolganova, T. V., Skryabin, K. G.,* Appl. Biochem. Microbiol. **39** (2003) 152.
12. *Granot, I., Aharonowitz, Y., Freeman, A.,* Appl. Microbiol. Biotechnol. **27** (1988) 457.
13. *Hocknull, M. D., Lilly, M. D.,* Enz. Microb. Technol. **10** (1988) 669.
14. *Carrea, G., Riva, S., Bovara, R., Pasta, P.*, Enz. Microb. Technol. **10** (1988) 333.
15. *Silbiger, E., Freeman, A., Enz. Microb. Technol.* **13** (1991) 869.
16. *Liu, W. H., Horng, W. C., Tsai, M. S.,* Enz. Microl. Technol. **18** (1996) 184.
17. *Angelova, B, Schmauder, H. P.,* J. Biotechnol. **67** (1999) 13.
18. *Cruz, A., Fernandes, P., Cabral, J. M. S., Pinheiro, H. M.,* J. Mol. Catal. B: Enzym. **11** (2001) 579.
19. *Cruz, A., Fernandes, P., Cabral, J. M. S., Pinheiro, H. M.,* Enz. Microb. Technol. **34** (2004) 342.
20. *Hesselink, P. G. M., Vliet, S ., Vries, H., Witholt, B.,* Enz. Microb. Technol. **11** (1989) 398.
21. *Schlosser, D., Irrgang, S., Schmauder, H. P.,* Appl. Microbiol. Biotechnol. **39** (1993) 16.
22. *Fernandes, P., Cabral, J. M. S., Pinheiro, H. M.,* J. Mol Catal. B: Enzym. **5** (1998) 307.
23. *Dias, A. C. P., Cabral, J. M. S., Pinheiro, H. M.,* Enz. Microb. Technol. **16** (1994) 708.
24. *Cabral, J. M. S., Aires-Barros, M. R., Pinheiro, H., Prazeres, D. M. F.,* J. Biotechnol. **59** (1997) 133.
25. *Llanes, N., Fernandes, P., Leon, R., Cabral, J. M. S., Pinheiro, H. M.,* J. Mol. Catal. B: Enzym. **11** (2001) 523.
26. *Wendhausen, R., Frigato, M., Fernandes, P., Carvalho, C. C. C. R., Cruz, A., Pinheiro, H. M. , Cabral, J. M. S.*, J. Mol. Catal. B: Enzym. **32** (2005) 61.
27. *Phase, N., Patil, S.,* World J. Microbiol. Biotechnol. **10** (1994) 228.
28. *Kutney, Y., Milanova, R., Vasilev, C., Stefanov S., Nedelcheva, N.,* 1999 PCT. Int. Appl. WO 99 49, 075, C.A.(1999) **131** 242083v.
29. *Sih, C.J., Tai, H. H., Tsang Y. Y., Coombe R. G.,* Biochem. **7** (1968) 808.