

Immobilization of *Mycobacterium* sp. NRRL B-3805 Cells onto Radiation Crosslinked PVA/PVP Hydrogels for Production of Androstenones from β -Sitosterol

Hala Abdel Salam Amin, Abeer Abd El-Hadi, Sayeda Saleh Mohamed

Chemistry of Natural and Microbial Products Department, National Research Center,
Dokki, Cairo, Egypt.

Abstract: The use of hydrophilic supports, as an immobilization matrix for mycobacterial cells, in a bioconversion system where hydrophobic species are present, such as sterols and mycobacterial cells, was evaluated. Several homo- and co-polymers prepared by γ -irradiation were tested for cells surface adhesion. Immobilized mycobacterial cells onto poly (vinyl alcohol) (PVA)/ poly (vinyl pyrrolidone) (PVP) copolymer hydrogel led to the best bioconversion estimates. The highest yield of AD was observed using PVA/PVP copolymer of composition (8:2, w/w) polymerized at 20kGy γ -irradiation dose. Under these conditions, almost the same product yield was obtained after only 24h incubation period compared with 48h in case of the free cells. Moreover, PVA/PVP mycobacterial beads could be used for 10 successive biotransformation cycles with a maximum product output of about 82%, which was of higher magnitude than that obtained by the free mycobacterial cells (76.5%). This work clearly highlights the ability of hydrogels to serve as an efficient immobilization matrix for mycobacterial cells to convert β -sitosterol to AD.

Key words: β -Sitosterol, androstenones, *Mycobacterium* sp. NRRL B-3805, immobilization, hydrogels

INTRODUCTION

Although steroid drugs represent only a small part (2.5%) of the world market of pharmaceuticals, the worldwide pharmaceutical industry needs more than 2000 tons per year of steroid raw materials (Schmid *et al.*, 2001). It is known that stigmasterol has been a suitable raw material for chemical synthesis of pregnane derivatives on an industrial scale because the C₂₄, C₂₅ double-bond promotes the chemical degradation of the side chain of the steroid skeleton. At the same time a large amount of β -sitosterol accumulated as a waste material during the production of stigmasterol (Szentirmai, 1990). Because of the successful utilization of *Mycobacterium* sp. mutants, to degrade the side-chain of sterols to produce 17-keto-steroids mainly; androsta-4-ene-3,17-dione (AD) and androsta-1,4-dien-3,17-dione (ADD) (Marsheck *et al.*, 1972), β -sitosterol now represents one of the most economical, inexpensive raw materials. AD and ADD are well-known pro-hormone substances and key precursors of pharmaceutical steroids.

Mycobacterium sp. NRRL B-3805 has been successfully used for the specific side chain cleavage of β -sitosterol to produce AD and ADD as side product (Baccaro *et al.*, 1995). Most of the biotransformations are traditionally carried out using free growing cells in aqueous media. Due to the presence of mycolic acids in the cell envelope, this bacterium demonstrates hydrophobic behavior and frequently forms cell clumps in aqueous media (Lee and Liu, 1992). Inhibitory effects of substrate and products also reduce the effectiveness of the transformation reaction (Lee and Liu, 1992). Cell immobilization has been attempted to alleviate the inhibitory effects of the product and substrate (Flygare and Larsson, 1987; Lee and Liu, 1992). Several carriers have been used to immobilize mycobacterial cells with β -sitosterol side chain cleavage activity. These include the adsorption on celite (Llanes *et al.*, 2001), chrysotile (Wendhausen *et al.*, 2005), or silicone rubbers (Claudino *et al.*, 2008). Still, only batch mode of operation, using celite-adsorbed mycobacterial cells, has been reported (Fernandes *et al.*, 1998a,b; Llanes *et al.*, 2001) and the repeated use of this immobilized biocatalyst in successive batches did not prove effective (Llanes *et al.*, 2001).

Hydrogels are polymeric networks, which absorb and retain water without dissolving. This property makes them interesting materials as carriers for immobilization of bioactive compounds as alternatives to others successfully used (Rosiak and Yoshii, 1999). PVA is a polymer with exceptional properties such as water

Corresponding Author: Hala Abdel Salam Amin, Natural and Microbial Products Chemistry Department, National Research Center, Dokki, Cairo, Egypt.
E-mail: halaamin2007@yahoo.com,
Phone: +202-33464472, Fax: +202-37622603

solubility, biodegradability, biocompatibility, non-toxicity and non-carcinogenicity that possesses the capability to form hydrogels by chemical or physical methods (Patachia *et al.*, 2009).

The objective of the present work was to probe the feasibility of mycobacterial cell adsorption onto homo- or copolymer hydrogels with the purpose of increasing the efficiency of β -sitosterol transformation to AD. High operational stability of the immobilized cells to be used in repeated batch process was also outlined. Thus, the effect of polymer composition, total absorbed γ -irradiation dose, and bioconversion time were looked into and the feasibility of reutilization was tentatively explored.

MATERIALS AND METHODS

Materials:

β -Sitosterol (60%) was obtained from Fluka (Buchs, Switzerland). 4-Androstene-3,17-dione (AD), 1,4-androstenediene-3,17-dione (ADD) and testosterone (TS) were reference grade from Sigma (USA). Polyvinyl alcohol (PVA) with degree of polymerization of 14000 and polyvinyl pyrrolidone (PVP) with an average molecular weight of 13000000 K85-95 were purchased from Merck (Germany). 2-hydroxyethyl methacrylate (HEMA) was supplied by BDH (Poole, UK). Poly ethylene glycol with a molecular weight of 400 was obtained from Sigma (USA). All other chemicals were of analytical or high-performance liquid chromatography (HPLC) grade and were procured from various standard sources.

Preparation of hydrogels:

Aqueous solutions of PVA (10%, w/v) and PVP (10%, w/v) were prepared in a water bath at 90°C for 50 min. PVA/PVP co-polymers were prepared by mixing PVA and PVP solutions with each other at different compositions (10:0, 9:1, 8:2, 6:4, 4:6, 0:10 w/w, respectively). HEMA monomer was mixed with distilled water and ethanol (2:3, v/v) to yield a final concentration of 10%. The aqueous solutions were poured in glass tubes. The nitrogen gas was passed through the solutions for 24 h to remove the dissolved oxygen. They were irradiated at -78°C with γ -rays from a Co^{60} source for 8h with a dose rate of 20 kGy/h (unless otherwise stated). The resultant homo- and co-polymer carriers were cut into discs, approximately 3-5mm in diameter, and shaken with an excess amount of water for 2 days in order to be fully swollen. The swollen carriers were sterilized by autoclaving at 121°C for 40 min. The sterilized carriers were immersed separately into the nutrient medium for 2 days to imbibe the medium (Abd El-Hadi, 2003).

Cell Growth and Immobilization:

Mycobacterium sp. NRRL B-3805 was maintained on potato dextrose agar slants (42 g/L) at room temperature. Unless stated otherwise, the tested microorganism was grown either in the presence or absence of hydrogels by adding an inoculum to 250 ml Erlenmeyer flasks, containing 50 ml of medium III at 30°C for 48 h. Cells were immobilized by adsorption onto hydrogels, added to the growth medium in a weight to volume ratio of 5g wet weight/50 ml medium. Immobilized cells were harvested in an adequate sieve at the late exponential growth phase, thoroughly washed with pH 7 potassium phosphate buffer (0.2 M), and stored at -20°C until use. Free cells were harvested by centrifugation (5 min, 4°C, 5000 rpm) in the late exponential growth phase, thoroughly washed with pH 7 potassium phosphate buffer (0.2 M). The wet cell paste (roughly 120 mg dry cell weight/g) was either immediately used in bioconversion trials or stored at -20°C until use (Staeblel *et al.*, 2004).

Bioconversion:

Unless otherwise stated, experiments were performed in 250 ml Erlenmeyer flasks, containing 50 ml of medium III supplemented with 15 mg solution of β -sitosterol in 1 ml ethanol, either with free (2 g wet cell paste) or immobilized cells. Bioconversion was extended to 48 h, in a rotary shaker (150 rpm) at 30°C. At the end of the transformation period, medium was extracted with twice its volume with chloroform. On occasion, when immobilized biocatalysts were used, solid and aqueous phase were recovered and extracted separately with chloroform. The extraction was repeated three times. The organic phase was collected and evaporated to dryness. The dried solids (test material) were then dissolved in chloroform and assayed for AD, ADD and TS using TLC and/or high performance liquid chromatography (HPLC).

Transformation media (g/l, w/v):

Medium I: $(\text{NH}_4)_2\text{SO}_4$, 10; Na_2HPO_4 , 4.5; KH_2PO_4 , 3.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2; Triton-X100, 0.5 (pH 6.5) (Wang *et al.*, 2006).

Medium II: (NH₄)₂SO₄, 5; Na₂HPO₄, 4.5; KH₂PO₄, 3.4; MgSO₄·7H₂O, 0.5 glycerol, 10; Triton-X100, 0.5 (pH 6.5) (Wang *et al.*, 2006).

Medium III: glucose, 10; peptone, 10; NH₄COO CH₃, 1.5; MgSO₄·7H₂O, 0.2; K₂HPO₄, 0.4; KH₂PO₄, 0.8; FeSO₄·7H₂O, 0.005mg; ZnSO₄·7H₂O, 0.002; MnCl₂·4H₂O, 0.0005 (pH 6.5) (Huang *et al.*, 2006).

Medium IV: yeast extract, 10; glycerol, 10; MgSO₄·7H₂O, 0.14; sitosterol, 0.5; Tween 20, 0.8 in pH 7 potassium phosphate buffer (20 mM) (Staebler *et al.*, 2004).

Medium V: fructose, 10.0; NH₄Cl, 2.0; MgSO₄·7H₂O, 0.14; K₂HPO₄, 2.11; KH₂PO₄, 0.75; Tween 20, 0.8 (pH 7.2) (Fernandes *et al.*, 1998a).

Analytical methods:

For TLC, aliquots were applied to Kieselgel 254 (Merck, Germany) plates. These were developed in benzene/ethylacetate/acetone (80:20:10, v/v) and visualized under UV light (254 nm). β-sitosterol was visualized using spraying by 10% sulphuric acid solution in ethanol and heating at 120°C for 10 min.

For HPLC, a Serva octadecyl-daltosil 100 column (250mm long, 4.6mm i.d., 5 μm film) was used, with acetonitrile/H₂O/glacial acetic acid (57:43:0.07, v/v) as a mobile phase (30°C) at a flow rate of 1cm 3min⁻¹, with detection at 240 nm. AD, ADD and TS were used as standards (Donova *et al.*, 2005). Conversion of β-sitosterol into AD, ADD and TS was estimated as follows (Perez *et al.*, 2005):

$$\text{Conversion (\%)} = \frac{[\text{weight androstenone/MW androstenone}]}{[\text{weight } \beta\text{-sitosterol /MW } \beta\text{-sitosterol}]} \times 100$$

where MW is the molecular weight.

Statistical analysis:

All the experiments were performed in triplicate, and the values expressed as the means of duplicate measurements of three independent samples. Data were analyzed by one-way analysis of variance (ANOVA) using MedCalc Software-version 11.2. The least significant differences at confidence level of 5% (LSD .05) and 1% (LSD .01) were calculated to compare the influence of different treatments on the mycobacterial cells activity.

RESULTS AND DISCUSSION

Optimization of β-sitosterol Bioconversion Conditions by Free Cells:

Cell culture media and bioconversion time course are important factors to influence the enzyme biocatalysis capabilities. *Mycobacterium* sp. NRRL B-3805 was separately cultivated in five different nutritive media (I-V) supplemented with 10 mg β-sitosterol dissolved in 1 ml ethanol (Table 1). Comparing the retention time of the HPLC chromatograms obtained from biotransformation products to the retention time of authentic reference samples, AD and TS were detected in the tested media. However, ADD was produced only in three media (III, IV and V). AD was the major product in the tested media except media I and II, where in TS was the major product. The detection of AD as a major product in the most tested media was in agreement with that obtained by Marsheck *et al.* (1972), Cruz *et al.* (2001), Llanes *et al.* (2001) and Staebler *et al.* (2004) for *Mycobacterium* sp. NRRL B-3805. The highest yield of TS (9.2%) was achieved with medium I. A single-step microbial transformation process for the production of TS from phytosterol via AD by a mutant of *Mycobacterium* sp. or *Mycobacterium* sp. VKM Ac-1816D has been established (Egorova *et al.*, 2009; Lo *et al.*, 2002). Medium III maintained the highest total molar conversion yield (32%) accompanied with the maximal AD yield (20.6%). This justifies its selection as the most suitable medium in the subsequent studies. These results may be interpreted in view that the constitution of this medium affects positively the enzyme (s) carrying out the formation of these products.

Fig. 1 shows the bioconversion time course of β-sitosterol conversion by mycobacterial free cells in medium III. The maximum accumulation of AD and ADD (1.42 and 0.26 mg, respectively) occurred after 48 h of cultivation, thereafter it decreased gradually until the end of experiment time (144 h). The conversion of β-sitosterol to AD was about 21%. In contrast, the highest yield of TS was recorded at 144 h, at which β-sitosterol was completely consumed and ADD was lost completely. These results are in a good agreement with various reports those declared that the reduction of AD to TS was catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) in the *Mycobacterium* sp. (Peltoketo *et al.*, 1999). The supply of reducing power,

NADH, from the metabolism of glucose was necessary for the reduction of AD to TS (Huang *et al.*, 2006).

In order to enhance the process productivity; different substrate (β -sitosterol) concentrations ranging from 0.1 to 0.4 g/l were supplemented to the culture medium at the inoculation time. Table 2 shows that the substrate concentration allowing for the maximal total bioconversion yield (41.8%) was 0.3 g/l, and this was accompanied by the highest molar yield of AD (28.5%). The bioconversion values were however decreased at higher substrate doses, suggesting substrate inhibition effect. This accords with results obtained by Llanes *et al.* (2001) who observed substrate inhibition of AD production by *Mycobacterium* sp. NRRL B-3805 cells immobilized on Celite for sitosterol concentrations above 6 mM. Also, this might be due to the poor solubility of the substrate in the aqueous media or the limit of the amount and activity of the enzyme.

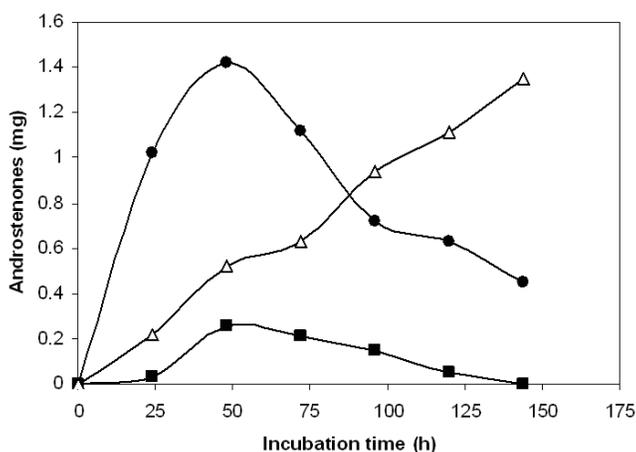


Fig. 1: Time course of the accumulation of androstenones during β -sitosterol conversion by *Mycobacterium* sp. NRRL B-3805 free cells.

Bioconversion was performed in medium III supplemented with 0.1g/l β -sitosterol at $30^{\circ}\text{C} \pm 2$ and 150rpm. Androstenones (AD: ●, ADD: ■, TS: △): mean of three values which were calculated in mg/50ml medium.

Several studies indicated that the sterols are hydrophobic substances with low water solubility in the media and this led to poor mass transfer (Cruz, *et al.*, 2001; Staebler *et al.*, 2004; Wang *et al.*, 2006). An attempt was performed to enhance mass transfer of sterol substrate in aqueous media by using various solubilizing agents and nonionic surfactants, i.e. ethanol, poly ethylene glycol, Triton X-100, Tween 80 and Tween 20, which were added, in the present investigation, to the medium in concentration of 0.08%, v/v. Fig. 2 shows that poly ethylene glycol supported the highest AD molar yield (34%) as well as the highest total conversion yield (50.9%). Similarly, Tween 80 and Tween 20 supported good biotransformation activities. The reason for the improvement in biotransformation by these agents is that they increased the transport across cell barrier by enhancing the substrate solubilization in media surrounding the cell, consequently the concentration gradient. Previous studies (Fernandes *et al.*, 1998a,b; Li *et al.*, 2009) showed that the addition of surface active agents to the fermentation broth while feeding can promote the dispersion of the substrate in the aqueous solution, which may greatly increase the conversion, because the substrate particle size is a key factor in the process. However, Triton X-100 exerted a deleterious effect on the bioconversion process. This may be due to its toxic effect on cells, as low cell growth was observed. The above findings suggested that in choosing a suitable solubilizing agent, a compromise must be made between its substrate solubilization capability and its biocompatibility with cells. To determine the optimum level of poly ethylene glycol, different levels ranging from 0.04 to 0.2 g% were individually added to the transformation medium. It was demonstrated that (data not shown) as the amount of poly ethylene glycol increased the total molar conversion yields were parallel increased until reached maximum (61.6%) at 0.12 level. Under this optimal condition, AD, ADD and TS productivities reached 4.31, 0.71 and 1.36 mg, respectively.

Results in Fig. 3 confirm that addition of D-alanine, L-asparagine and glycine enhances the bioconversion process and led to relatively higher AD, ADD and TS yields compared with control treatment (without amino acids addition). The highest total conversion yield of 76.5% was achieved using D-alanine. Sedlaczek *et al.* (1999) demonstrated that glycine enhances β -sitosterol penetration through the mycobacterial cell wall due to

disturbing the integrity of the cell wall components (peptidoglycan and mycolic acids) responsible for the permeability barrier in mycobacteria. The rate of transformation of β -sitosterol to 4-androsten-3,17-dione (AD) by *Mycobacterium vaccae* increased considerably in the presence of D,L norleucine and m-fluorophenylalanine. These compounds inhibit the biosynthesis of the complex lipids in the cell wall outermost layer (Rumijowska *et al.*, 2000). On the other hand, lower bioconversion values were observed using L-cysteine and L-leucine.

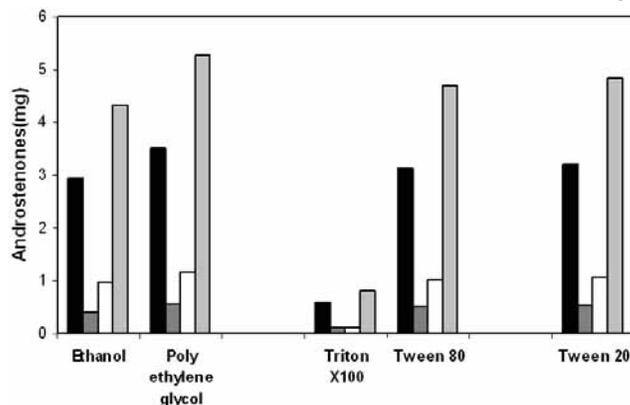


Fig. 2: Effect of organic solvents and nonionic surfactants on β -sitosterol bioconversion by *Mycobacterium* sp. NRRL B-3805 free cells.

Bioconversion was performed in medium III separately supplemented with 0.8g/l organic solvents or surfactants for 48h at $30^{\circ}\text{C} \pm 2$ and 150rpm. Androstenones ((AD: ■,ADD: ■,TS: □, Total: ■)): mean of three values which were calculated in mg/50mL medium.

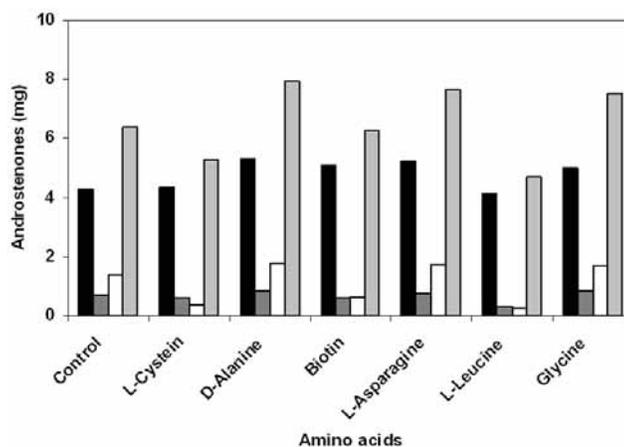


Fig. 3: Effect of amino acids on β -sitosterol bioconversion by *Mycobacterium* sp. NRRL B-3805 free cells. Bioconversion was performed in medium III (50ml) separately supplemented with 5mg of different amino acids for 48h at $30^{\circ}\text{C} \pm 2$ and 150rpm. Androstenones ((AD: ■,ADD: ■,TS: □,Total: ■)): mean of three values which were calculated in mg/50mL medium.

Immobilization Using Different Carriers:

Given the hydrophobic nature of both substrate and mycobacterial cells the development of an immobilized cell system presents some particular constraints (Sedlaczek *et al.*, 1999). Mass transfer limitations may take place if hydrophilic supports are used, such as hydrogels used in cell immobilization due to inadequate partition of the lipophilic substrates into the gel (Freeman and Lilly, 1998). The diffusion phenomena can markedly decreased by cell immobilization on the surface of the hydrogel (Le-Tien *et al.*, 2004). For this purpose, PVA, PVP, PVA/PVP and HEMA hydrogels were immersed separately into the optimized fermentation medium containing free mycobacterium cells. The cells were adsorbed and became immobilized. As shown in Table 3, higher bioconversion values were consistently observed when PVA and PVP homo- and copolymers (PVA

based hydrogels) were used as immobilization matrix, as compared to HEMA polymer. For non-ionic hydrogels such as PVA, PVP and HEMA, swelling is controlled by the hydrophilicity of the polymers or monomers (Oztop *et al.*, 2002). The hydrophilicity of PVA and PVP hydrogels is much higher than that of HEMA based hydrogel. The higher swelling of the hydrogels permitted the presence of more nutrient medium and cells inside of the hydrogel. Although the highest bioconversion yield (58.2%) was recorded using PVP homo-polymer hydrogel, PVA/PVP co-polymer is more preferable due to its higher mechanical strength (Baccaro *et al.*, 1995). The toughness of PVP hydrogel is improved by the addition of PVA, so that PVA/PVP hydrogel was selected for β -sitosterol bioconversion.

Optimization of β -sitosterol Bioconversion *Mycobacterial Cells Adsorbed onto PVA/PVP hydrogels:*

PVA hydrogels containing various amounts of PVP were produced by γ -irradiation at dose of 20 kGy. The effect of different compositions of PVA/PVP on β -sitosterol conversion is shown in Fig. 4. AD yield as well as the total bioconversion yield reached maximum values (4.45 mg and 63.8%, respectively) when cells were adsorbed on PVA/PVP hydrogel of composition (8:2, w/w). Increasing of the PVP content beyond the later concentration led to a decrease in the bioconversion values. The cross-linking of PVA hydrogel with such a high hydrophilic PVP polymer seems to change the property of the polymer matrix to be a more porous gel. This increased the diffusion rate of substrate and products into and from the polymer matrix giving higher bioconversion yields. On the other hand, cross-linking with PVP polymer concentrations higher than 20% gave a hydrogel with high swelling property, low strength and low gel content. These seem to be undesirable for the bioconversion process (Abd El-Hady, and Abd El-Rehim, 2004).

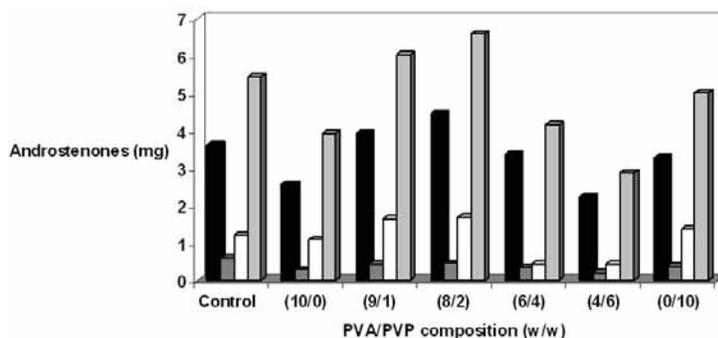


Fig. 4: Effect of PVA/PVP composition on β -sitosterol bioconversion by immobilized *Mycobacterium* sp. NRRL B-3805.

Copolymers (10%) were polymerized at 20kGy. Control: using PVA/PVP hydrogel of composition (5/5). Androstenones (AD: ■,ADD: ▒,TS: □,Total: ▓): mean of three values which were calculated in mg/50mL medium.

The time course of β -sitosterol bioconversion by mycobacterium cells adsorbed on PVA/PVP hydrogel is shown in Fig. 5. The reaction time was shortened compared with that when free cells were used (Fig. 1), and the maximum total conversion yield was slightly lower by about 6%. The highest total products yield of 7.26 mg/50ml, which corresponds to molar conversion yield of 70.1% was reached after 24 h. Thus, adsorption of mycobacterial cell on PVA/PVP hydrogel led to an increase in the reaction rate but did not increase the final conversion efficiencies. This behavior suggests that cell adsorption onto the hydrogel during growth does not affect significantly the profile of β -sitosterol side-chain cleavage.

Ionizing radiation such as cobalt-60 gamma radiation provides a clean method for the production and modification of polymers. No chemicals or catalysts have to be added to the reaction matrix. The polymerization is achieved by free radicals (occasionally ions) created in the material and therefore no chemicals or catalysts remain in the material after radiation (Oztop *et al.*, 2002). Bioconversion of β -sitosterol to androstenones by immobilized mycobacterium cells is greatly affected by the total γ -irradiation dose adsorbed by PVA/PVP hydrogels. Table 4 shows that the maximal total bioconversion yield (75%) was obtained when γ -radiation of 20 kGy was used for PVA/PVP cross-linking process. However, the cross-linking at lower or higher radiation doses (10, 15, 25 kGy) affected the conversion process adversely. At low irradiation dose, the degree of crosslinking was low resulting in a polymer with a weak mechanical strength. Therefore, the adsorbed mycobacterial cells onto hydrogel matrix and in its pores were of lower magnitude

and this in turn led to less bioconversion yields (45.3 and 61% at 10 and 15 kGy, respectively). As the irradiation dose increases, the polymer free radicals increase and consequently the degree of crosslinking and the adsorbed cells increase. Cross-linking at irradiation dose of 25 kGy higher than the optimum (20 kGy) produced a more rigid polymer with strong mechanical strength. This reduced the copolymer swelling values and subsequently the diffusion rate of the substrate and products decreased resulting in reduction in the bioconversion estimates (Yoshii *et al.*, 1999).

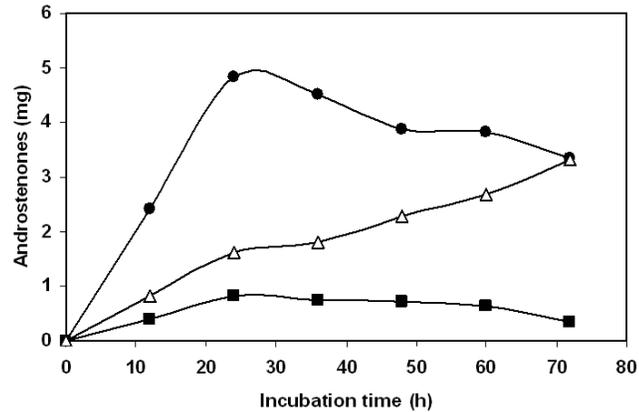


Fig. 5: Time-course of the accumulation of androstenones during β -sitosterol conversion by immobilized *Mycobacterium* sp. NRRL B-3805.

Cells adsorbed on PVA/PVP hydrogel of composition (8/2) were incubated in medium III supplemented with 0.1g/l β -sitosterol at 30°C \pm 2 and 150rpm. Copolymer (10%) was irradiated at 20kGy. Androstenones (AD: ●, ADD: ■, TS: Δ): mean of three values which were calculated in mg/50mL medium.

Table 1: Effect of the medium composition on β -sitosterol bioconversion by *Mycobacterium* sp. NRRL B-3805 free cells.

Medium	Dry weight (g%)	Androstenones(mg)				Total conversion (%)
		AD	ADD	TS	Total	
I	0.12	0.08	0	0.64	0.72	10.4
II	0.15	0.32	0.11	0.49	0.81	12.5
III	0.53	1.42	0.26	0.52	2.2	32
IV	0.32	0.8	0.18	0.26	1.24	18
V	0.41	1.05	0.16	0.4	1.45	21.1

The cultivation media I-V (for composition see section materials and methods) were supplemented with 0.1g/l β -sitosterol. Media (50ml) were inoculated with 2g wet cell paste and incubated for 48h at 30°C \pm 2 and 150rpm. Androstenones (mg): mean of three values which were calculated in mg/50 ml medium. AD: androsta-4-ene-3,17-dione, ADD: androsta-1,4-dien-3,17-dione and TS: Testosterone.

Table 2: Effect of substrate concentration on β -sitosterol bioconversion by *Mycobacterium* sp. NRRL B-3805 free cells.

b-Sitosterol (g/L)	Androstenones (mg)				Total conversion (%)
	AD	ADD	TS	Total	
0.1	0.79	0.05	0.16	1	28.3
0.2	1.42	0.26	0.52	2.2	32
0.3	2.95	0.4	0.98	4.33	41.8
0.4	2.2	0.06	0.6	2.86	20.7

The biotransformation was performed in medium III for 48h at 30°C \pm 2 and 150rpm with 2g wet cell paste. Androstenones (mg): mean of three values which were calculated in mg/50ml medium.

Table 3: Effect of different carriers on β -sitosterol bioconversion by immobilized *Mycobacterium* sp. NRRL B-3805.

Carrier	Dry weight (g/g carrier)	Androstenones (mg)				Total conversion (%)
		AD	ADD	TS	Total	
PVA	0.23	3.47	0	1.73	5.2	50.2
PVP	0.26	4.02	0.67	1.34	6.03	58.2
PVA/PVP (5/5)	0.29	3.63	0.6	1.21	5.44	52.6
HEAMA	0.15	1.43	0	0.71	2.14	20.7
Control (free cells)	-	5.32	0.84	1.76	7.92	76.5

Hydrogels polymerized by γ -irradiation at 20kGy. Bioconversion was performed by 5g_{wet weight} immobilized cells/50ml of medium III, for

48h at 30°C ±2 and 150rpm. Androstenones (mg): mean of three values which were calculated in mg/50mL medium.

Table 4: Effect of the irradiation doses on β-sitosterol bioconversion by immobilized *Mycobacterium* sp. NRRL B-3805.

Gama ray dose (kGy)	Dry weight (g/g carrier)	Androstenones(mg)				Total conversion (%)
		AD	ADD	TS	Total	
10	0.15	3.12	0.52	1.04	4.69	45.3
15	0.28	4.2	0.7	1.4	6.31	61
20	0.33	5.2	0.86	1.72	7.78	75.2
25	0.25	4.84	0.81	1.61	7.26	70.1

Cells adsorbed on PVA/PVP hydrogel of composition (8/2) incubated in medium III for 30°C ±2 and 150rpm. Androstenones (mg): mean of three values which were calculated in mg/50ml medium.

Reusability of PVA/PVP immobilized cells:

Poly (vinyl alcohol) (PVA)-based hydrogels have become widely spread for cell immobilization primarily due to the very high operational stability of the gel matrix (Lozinsky and Plieva, 1998; Patachia *et al.*, 2009). In order to evaluate the feasibility of biocatalyst reutilization, PVA/PVP adsorbed cells (3-5 mm particle size) obtained under the described optimal conditions were used in 10 repeated batch biotransformation processes. Results given in Fig. 6 indicate that at the end of the first batch (24 h) AD reached 4.88 mg, which representing about 92% of the maximum yield of AD produced by the free cells after 48h. In the next cycle, AD yield was markedly increased to reach about 5.7mg. This was accompanied by high total conversion yield (82.1%), which was of higher magnitude than that obtained by the free mycobacterial cells (76.5%). Despite the shaking conditions, bead shape was not affected even after consecutive biotransformation cycles. Moreover, adsorption onto PVA/PVP hydrogel surface helped to stabilize the biocatalyst, resulting in operational stability when performing consecutive production cycles up to the 6th cycle (conversion yield > 50%). However, the bioconversion estimates with the prolonged utilization of the immobilized biocatalyst were decreased gradually up to the 10th run. This effect was assigned to the lyses of the cells, and thus the density of the immobilized cells becomes lower and so led to lower in the cells multiplication and so decreases in the activity of the immobilized mycobacterial cells (Silbiger and Freeman, 1991). In the overall, PVA/PVP immobilized mycobacterial cells showed higher stability for β-sitosterol conversion compared to mycobacterial cells adsorbed on celite (Llanes *et al.*, 2001) or silicone rubbers (Claudino *et al.*, 2008).

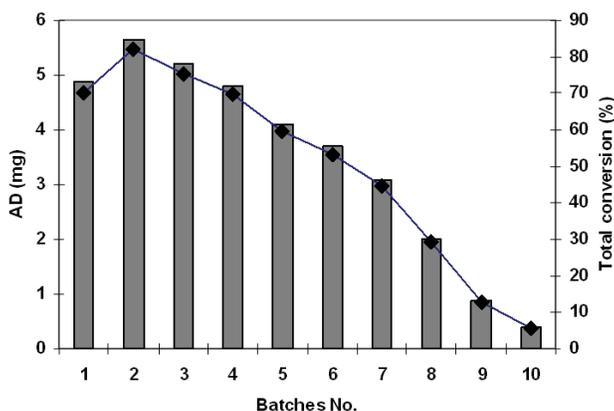


Fig. 6: Reusability of PVA/PVP immobilized.

Bioconversion was performed using 5g immobilized biocatalyst at the first batch. After each batch (24h), immobilized cells were washed with phosphate buffer and resuspended in 50ml medium III supplemented with 15mg β-sitosterol. AD (◆) and Total conversion (■) were mean of three values which calculated in 50 ml medium.

The previously mentioned achievements may contribute to the improvement of the biotechnology basis of sterols bioconversion to the medicinally important C-19 steroids, namely at the level of operational stability. This approach is considered as the first report explored the feasibility of applying immobilized mycobacterial cells onto PVA based hydrogels in β-sitosterol biotransformation. This further highlights the potentiality of such a system, especially regarding the development of an industrial bioprocess consisting in an immobilized-cell stirred tank bioreactor for continuous production of AD from β-sitosterol.

REFERENCES

- Abd El-Hadi, A., 2003. Production of prednisolone by *Bacillus pumilus* E601 cells incorporated in radiation induced poly(vinyl alcohol g-2 hydroxyethylmethacrylate) cryogels. *Process. Biochem.*, 38(12): 1653-1657.
- Abd El-Hady, A. and H.A. Abd El-Rehim, 2004. Production of Prednisolone by *Pseudomonas oleovorans* cells incorporated into PVP/PEO radiation crosslinked hydrogels. *J Biomed. Biotechnol.*, 4: 219-226.
- Baccaro, S., L.A. Pajewski, G.Scoccia, R.Volpe and J.M. Rosiak, 1995. Mechanical properties of polyvinylpyrrolidone (PVP) hydrogels undergoing radiation. *Nuclear Instruments and Methods in Physics Research., B*, 105: 100-102.
- Claudino, M.J.C., D. Soares, F.Van Keulen, M.P.C. Marques, J.M.S. Cabral and P. Fernandes, 2008. Immobilization of mycobacterial cells onto silicone- Assessing the feasibility of the immobilized biocatalyst in the production of androstenedione from sitosterol. *Bioresource. Technol.*, 99: 2304-2311.
- Cruz, A., P. Fernandes, J.M.S. Cabral and H.M. Pinheiro, 2001. Whole-cell biotransformation of b-sitosterol in aqueous-organic two-phase systems. *J. Mol. Catal. B: Enzym.*, 11: 579-585.
- Donova, M.V., S.A. Gulevskaya, D.V. Dovbnya and I.F. Puntus, 2005. *Mycobacterium* sp. mutant strain producing 9 α -hydroxyandrostenedione from sitosterol. *Appl. Microbiol. Biotechnol.*, 67: 671-678.
- Egorova, O.V., V.M. Nikolayeva, G.V. Sukhodolskaya and M.V. Donova, 2009. Transformation of C19-steroids and testosterone production by sterol-transforming strains of *Mycobacterium* spp. *J. Mol. Catal. B: Enzym.*, 57: 198-203.
- Fernandes, P., J.M.S. Cabral and H.M. Pinheiro, 1998a. Influence of some operational parameters on the bioconversion of sitosterol with immobilized whole cells in organic medium. *J. Mol. Catal. B: Enzym.*, 5: 307-310.
- Fernandes, P., J.M.S. Cabral and H.M. Pinheiro, 1998b. Stability of free and immobilized *Mycobacterium* sp. cells in aqueous and organic media. In: *Progress in Biotechnology 15-Stability and Stabilization of Biocatalysts*, Eds., Ballesteros A., F.J. Plou, J.L. Iborra and P.J. Halling Elsevier, Amsterdam, pp: 625-630.
- Flygare, S. and P.O. Larsson, 1987. Steroid transformation using magnetically immobilized *Mycobacterium* sp. *Enzym. Microb. Technol.*, 9: 494-499.
- Freeman, A. and M.D. Lilly, 1998. Effect of processing parameters on the feasibility and operational stability of immobilized viable microbial cells. *Enzyme Microb. Technol.*, 23: 335-345.
- Huang, C.L., Y.R. Chen and W.H. Liu, 2006. Production of androstenones from phytosterol by mutants of *Mycobacterium* sp. *Enzyme. Microb. Technol.*, 39: 296-300.
- Lee, C.Y. and W.H. Liu, 1992. Production of androsta-1,4-diene-3,17-dione from cholesterol using immobilized growing cells of *Mycobacterium* sp. NRRL B-3683 adsorbed on solid carriers. *Appl. Microbiol. Biotechnol.*, 36: 598-603.
- Le-Tien, C., M. Millette, M. Lacroix and M.A. Mateecus, 2004. Modified alginate matrices for the immobilization of bioactive agents. *Biotechnol. Appl. Biochem.*, 39: 189-198.
- Li, J.H., Y.X. Guan, H.Q. Wang and S.J. Yao, 2009. Dehydrogenation of 11 α -hydroxy-16 α ,17-epoxyprogesterone by encapsulated *Arthrobacter simplex* cells in an aqueous/organic solvent two-liquid-phase system. *J. Chem. Technol. Biotechnol.*, 84: 208-214.
- Llanes, N., P. Fernandes, R. Leon, J.M.S. Cabral and H.M. Pinheiro, 2001. Conversion of b-sitosterol by *Mycobacterium* sp. NRRL B-3805 cells immobilized on Celite supports. *J. Mol. Catal. B: Enzym.*, 11: 523-530.
- Lo, C.K., C.P. Pan and W.H. Liu, 2002. Production of testosterone from phytosterol using a single-step microbial transformation by a mutant of *Mycobacterium* sp. *J. Ind. Microbiol. Biotechnol.*, 28: 280-283.
- Lozinsky, V.I. and F.M. Plieva, 1998. Poly (vinyl alcohol) cryogels employed as matrices for cell immobilization. 3. Overview of recent research and developments. *Enzyme. Microb. Technol.*, 23: 227-242.
- Marshech, W.J., S. Kraychy and R.D. Muir, 1972. Microbial degradation of sterols. *Appl. Microbiol.*, 23(1): 72-77.
- Oztop, H.N., A.Y. Oztop, Y. Isikver and D. Saraydin, 2002. Immobilization of *Saccharomyces cerevisiae* on to radiation crosslinked HEMA/AAm hydrogels for production of ethyl alcohol. *Process. Biochem.*, 37: 651-657.
- Patachia, S., C. Florea, C. Friedrich and Y.Thomann, 2009. Tailoring of poly(vinyl alcohol) cryogels properties by salts addition. *eXPRESS Polymer Letters*, 3(5): 320-331.
- Peltoketo, H., V. Luu-The, J. Simard and J. Adamski, 1999. 17- β -hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. *J. Mol. Endocrinol.*, 23: 1-11.

- Perez, C., A. Falero, B.R. Hung, S. Tirado and Y. Balcinde, 2005. Bioconversion of phytosterols to androstanes by mycobacteria growing on sugar cane mud. *J. Ind. Microbiol. Biotechnol.*, 32(3): 83-86.
- Rosiak, J.M. and F. Yoshii, 1999. Hydrogels and their medical applications. *Nucl. Inst. Meth. Phys. Res. B*, 151: 56-64.
- Rumijowska, A., A. Ziolkowski, M. Korycka, L. Sedlacek, 2000. Alterations in lipid composition of *Mycobacterium vaccae* cell wall outer layer enhance β -sitosterol degradation. *World J. Microbiol. Biotechnol.*, 16: 237-244.
- Schmid, A., J.S. Dordick, B. Hauer, A. Kiener, M. Wubbots and B. Witholt, 2001. Industrial biocatalysts today and tomorrow. *Nature*, 409: 258-268.
- Sedlacek, L., K. Lisowska, M. Korycka, A. Rumijowska, A. Ziolkowski, J. Dlugonski, 1999. The effect of cell wall components on glycine enhanced sterol side chain degradation to androstene derivatives by mycobacteria. *Appl. Microbiol. Biotechnol.*, 52: 563-571.
- Silbiger, E. and A. Freeman, 1991. Continuous Δ^1 - hydrocortisone dehydrogenation with in situ product recovery. *Enzyme Microb Technol.*, 13(11): 869-872.
- Staebler, A., A. Cruz, W. Goot, H.M. Pinheiro, J.M.S. Cabral and P. Fernandes, 2004. Optimization of androstenedione production in an organic-aqueous two-liquid phase system. *J. Mol. Catal. B: Enzym.*, 29: 19-23.
- Szentirmai, A., 1990. Microbial physiology of side chain degradation of sterols. *J. Ind. Microbiol.*, 6: 101-116.
- Wang, Z., F. Zhao, D. Chen and D. Li, 2006. Biotransformation of phytosterol to produce androsta-diene-dione by resting cells of *Mycobacterium* in cloud point system. *Process Biochem.*, 4: 557-561.
- Wendhausen, R., M. Frigato, P. Fernandes, C.C.C.R. Carvalho, A. Cruza, H.M. Pinheiro and J.M.S. Cabral, 2005. Chrysolite as a support for the immobilization of *Mycobacterium* sp. NRRL B-3805 cells for the bioconversion of β -sitosterol in an organic-aqueous two-liquid phase system. *J. Mol. Catal. B: Enzym.*, 32: 61- 65.
- Yoshii, F., Y. Zhanshan, K. Isobe, K. Shinozaki and K. Makuuchi, 1999. Electron beam crosslinked PEO and PEO/PVA hydrogels for wound dressing. *Radiat. Phys. Chem.*, 55: 133-138.