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Biotransformation of extracted digitoxin from *Digitalis lanata* by *Streptomyces*

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The biotransformation of digitoxin and some of its derivatives extracted from *Digitalis lanata* by *Streptomyces* isolated species was investigated. Cultures of a *Streptomyces* strain designated EUSA-2003B, isolated from an Egyptian soil sample, efficiently induced selective 12β-hydroxylation of the steroid aglycone of digitoxin (DT) and its α-acetyl and β-methyl derivatives. The transformation reaction was performed within a 5-day fermentation process, products were isolated and their aglycone moiety was obtained by acid hydrolysis and their structures were elucidated by ^{13}C and ^{1}H NMR. The biotransformation resulted mainly digoxin (DG, ~87%), meanwhile, digoxigenone (DGON, ~7.0%) was also afforded as a side product. The present study revealed that: 1-*Streptomyces* isolate EUSA2003B harbors its specific 12β-hydroxlase and has the capability to transform DT and it's α-acetyl and β-methyl derivatives into their corresponding digoxins at reasonable yields. 2-The minor structural differences in the trisaccharide side chain seemed ineffective on the transformational capability of this organism. 3-The *Streptomyces* might also possess a specific glycosidase that splits the saccharidic side chain beside another dehydrogenase that oxidizes C_3 at the steroid nucleus into its ketone form (DGON).

1. Introduction

Phytochemical reviews pointed out that cell cultures of *Digitalis lanata* (foxglove) synthesize and transform variable cardioactive substances known as digitalis cardenolides. Cardenolides are divided into 6 series (A to F), based on their "genin" part. Series A glycosides, like lanatoside A and digitoxin are the most abundant members, whereas type C, like digoxin are those clinically used (Kreis et al. 1990). Digoxin, is still used in the treatment of congestive heart failure by reducing the Na^+/K^+ ATPase activity and increasing, consequently, the cardiac muscle contractility (Matthew et al. 1974). Chemically, transformations of DTs into DGs have been tried in many laboratories using known strategies, but unfortunately, reported fastidious and time-consuming (Albrecht et al. 1982). The low therapeutic index of DT (Tanaka et al. 1990) which made DG clinically preferable has motivated several researchers to find a way of transforming DT into DG with sustaining the pharmacologic activity and high yield under mild fermentation conditions (Volkov 1994).Therefore, microbial biotransformation of DT-containing wastes, under optimized conditions, into their corresponding DGs is still a reasonable approach to achieve this goal.

Members of the cardiac glycosides are either transformed into cardioactive derivatives not known to occur in nature (Padua et al. 2005), or to valuable products in high yields (Pérez-Alonso et al. 2009). Therefore, application of microorganisms to induce stereo-specific structural modifications of cardenolides is still a promising approach for economic production of commercially required drugs. In fact, a few microbial species, mostly filamentous fungi, showed remarkable activity to hydroxylate the steran moiety (aglycone nucleus) in different positions including 1β (13); 5β (14); 7β and 12 β (Szelecky et al. 1980). However, early (Albrecht et al. 1982) and recent trials failed to achieve reasonable success using fungi for 12β-hydroxylation (Pádua et al. 2007). This study, as an alternative solution, is aiming to exploit cultures of a *Streptomyces* isolates for reproducible biotransformation of relatively toxic type "A" cardioactive drugs into their corresponding therapeutically desired forms (C type) via 12-β hydroxylation enzyme. Table 1

2. Investigations, result and discussion

Much interest was paid to the microbial transformation of toxic cardioactive drugs to their relatively less or non-toxic derivatives of high therapeutic index using cell cultures of plants (Kreis et al. 1990), bacteria (Fuska et al. 1987), *Streptomyces* (Baljet 1968) and fungi (Pádua et al. 2007). Filamentous fungi and *Streptomyces* present rates of biomass growth higher than those of plant cell cultures, what turns out their transformations more feasible for large scale applications. Nonetheless, little work was devoted to the of use soil *Streptomyces* isolates to perform this task. This prompted us to exploit soil *Streptomyces* for achieving the biotransformation of digitoxins into digoxins. After being subjected to transformation, DT and its α-acetyl and β-methyl derivatives, two compounds were obtained from the growing cultures of the *Streptomyces* EUSA2003B, namely digoxigenin and digoxigenone (Table 2), while the untransformed portions were detected as digitoxigenin. Digitoxin and its α-acetyl and β-methyl derivatives were

Table 1: Chemical structures of the cardenolides under investigation

Cardenolides	Abbreviation	R_1	R_2
Digitoxin	DT	H	Dox- β1,4- Dox- β1,4- Dox- β1-
Digoxin	DG	OH	Dox- β1,4- Dox- β1,4- Dox- β1-
α-Acetyldigitoxin	α -AcDT	H	α -AcDox- β1,4- Dox- β1,4- Dox- β1-
Digitoxigenin	DTG	H	H
β-Methyldigitoxin	β -MeDT	H	β-Me-Dox- β1,4- Dox- β1,4- Dox- β1-
Digoxigenin	DGG	OH	H

Dox = digitoxose; α –AcDox = α -Acetyldigitoxose; β- MeDox = β Methyldigitoxose

Table 2: Yield and retention times (R_t) of the assessed cardenolides

Cardenolides	Abbreviation	Yield[#] and R_t(min)
Digitoxin	DT	(24.7)
Digoxin	DG	~ 87% (22.3)
Digitoxigenin	DTG	(24.0)
Digoxigenin	DGG	~5.0% (25.9)
Digoxigenone	DGON	7.0% (19.4)

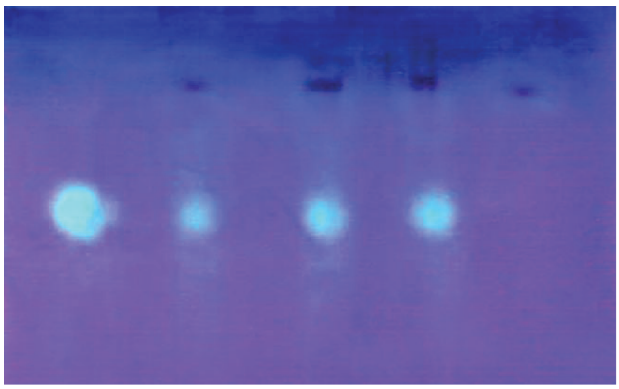
[#]Based on 10 mg cardinolide added to 2-day old growing culture of Streptomyces EUSA2003B allowed to grow for further 5 consecutive days

employed as substrates for biotransformation by the *Streptomyces* isolate after being individually added to 48 h growing cultures. Their transformation products were isolated, purified and acid-hydrolyzed liberating their modified aglycones to be quantified by HPLC (Table 1). Their purity and authenticity were comparatively attested by spectroscopic techniques. The transformation medium components lacking the organism (C-2 culture) were found not to affect the added cardenolides, since no transformation products were detected after the same period taken by the transforming *Streptomyces*. Besides digoxigenin (DGG), another 3-keto-12 β-hydroxy aglycone (Digoxigenone, DGON) was evolved since their proton chemical shifts were consistent with its structure (Table 3)**.** The elemental analysis of extracted digitoxin is showed 64.37% carbon, 8.43% hydrogen, and 27.19% oxygen. While elemental analysis of biotransformed digoxin is showed 63.06% carbon, 8.62% hydrogen, and 28.69% oxygen. Both digitoxin and digoxin give blue green fluorescence after spraying with acidic ferric chloride reagent. Then when exposed to UV light digitoxin gives orange fluorescence and digoxin gives blue fluorescence (Fig. 1). Concerning the physical properties, the ultraviolet spectrum showed one band at λ max 220 nm for biotransformed digoxin which satisfies the λ max of standard digoxin. On the other hand digitoxin gives one band at λ max 240 nm. Mass spectroscopy analysis showed molecular ion peak at 766, 781 and 782 for digitoxin, digoxin and biotransformed digoxin respectively which represent the molecular weight for the digitoxin, digoxin and bio transformed digoxin (Figs. 2–4). From the ^{1}H NMR spectrum of digoxigenin a complex profile and the only signals easily assigned were those of H-3α(δ 4.14), H-21(δ 4.90 and δ 4.80) and H-22 (δ 5.94) which confirmed the presence of C3-OH group and the α and β-unsaturated lactone ring. Singlets at δ 0.81 and δ 0.97 were

Table 3: ^{13}C and ^{1}H NMR assignments of digitoxigenin and the biotransformation products by *Streptomyces* EUSA-2003B

		Chemical shifts (δ/ppm)		
^{13}C	(^{1}H)	Digitoxigenin	Digoxigenin	Digoxigenone
1	1α;1β	29.6(1.49) (1.49)	29.6(1.49) (1.49)	37.8 (2.11) (1.45)
2	2 α;2 β	27.9(1.53) (1.53)	27.9 (1.53) (1.53)	37.9(2.53) (2.11)
3				
4	3 α	66.8(4.13)	66.7(4.14)	215.7(–)
	4 α;4 β	33.3(1.34) (1.89)	33.3(1.39) (1.94)	43.0(2.78) (1.95)
5	5 β	36.0(1.78)	36.0(1.84)	45.4(1.81)
6	6 α;6 β	26.5(1.87) (1.30)	26.3(1.90) (1.32)	27.8(1.93) (1.34)
7	7 α;7 β	21.2(1.25) (1.71)	21.6(1.26) (1.71)	22.4(1.31) (1.84)
8	8 β	41.8(1.56)	41.4(1.54)	42.1(1.66)
9	9α	35.5(1.62)	32.4(1.66)	34.3(1.91)
10		35.4	35.2	36.3
11	11α;11β	21.3(1.46) (1.46)	30.4(1.68) (1.27)	30.8(1.65) (1.34)
12	12α;12β	40.0(1.40) (1.53)	75.1(3.38) (–)	75.4(3.47) (–)
13		49.6	55.5	57.3
14		85.5	85.9	86.6
15	15α;15β	33.1(2.13) (1.71)	33.3(1.89) (1.82)	33.5(2.07) (1.77)
16	16α;16β	26.9(1.87) (2.16)	27.4(1.67) (2.16)	28.4(1.95) (2.15)
17	17α	51.0(2.79)	45.6(3.33)	47.0(3.35)
18		15.7(0.88)	8.9 (0.81)	9.9 (0.81)
19		23.7(0.96)	23.6(0.97)	22.8(1.05)
20		174.5(5.00)	174.2(4.90)	177.3(4.99)
21		73.4 (4.81)	73.6(4.81)	75.4(4.91)
22		117.6(5.87)	117.8(5.94)	117.8(5.99)
23		174.4	174.5	178.4

Values between brackets refer to chemical shifts of hydrogen.



A : Digoxin.

B : Biotransformed digoxin extracted from *BS-3* culture using authentic digitoxin.

C & D : biotransformed digoxin extracted from *BS-3* culture use plant digitoxin.

E : Digitoxin.

Fig. 1: TLC of digitoxin, digoxin and biotransformed digoxin

readily assigned indicating the presence of C_{18} and C_{19} methyl groups (Table 3). The hydroxylation position was unambiguously defined on the basis of the chemical shift changes recorded for digoxigenin (the acid-hydrolysis end product of transformed DT,α-acetyl and β-Me DT as well) which allowed to allocate 12 β-OH group. These results revealed that the bio transformed digoxin is identical to the authentic digoxin. Microbial transformation of digitoxin and some of its derivatives was successfully studied using *Streptomyces* sp., however, use of *Fusarium ciliatum* (Pádua et al. 2005) led to negative result which was hypothetically attributed to the insufficient impermeability of fungal cells to the DT as compared to vegetal cell membranes. In the present work, shortening of the reaction period to 5 days

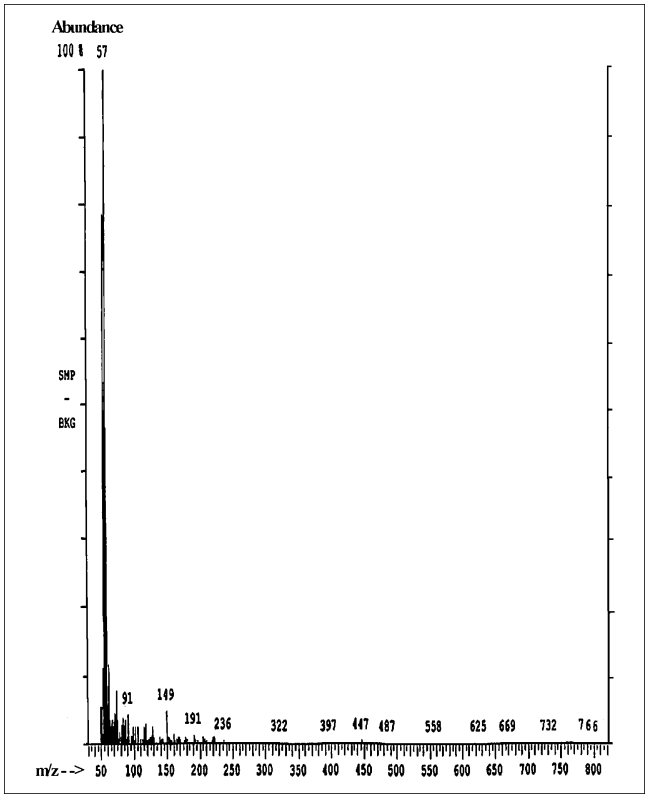


Fig. 2: Mass spectroscopy of digitoxin

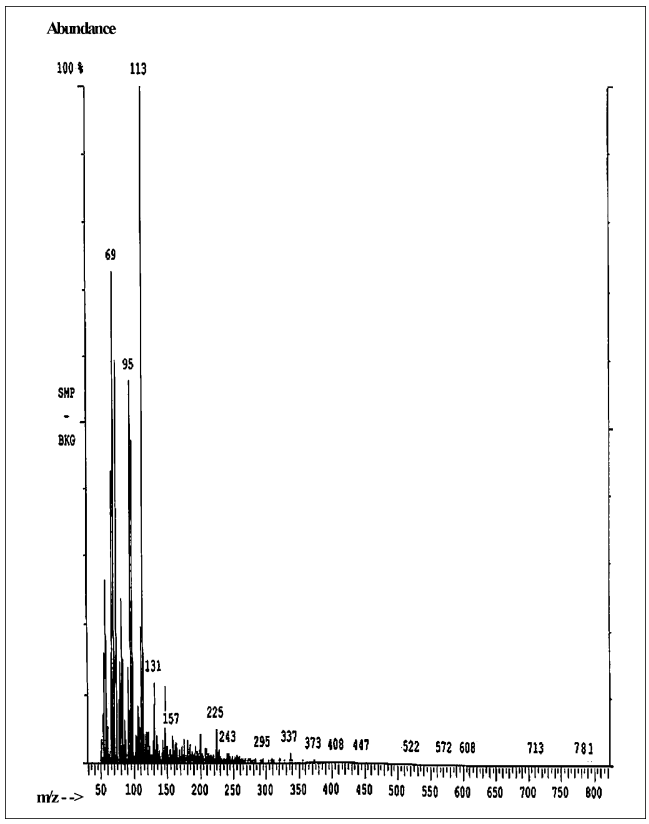


Fig. 3: Mass spectroscopy of digoxin

afforded reasonable yields of the 12β-hydroxylated products (DG, DGG, and DGON) which is explainable on the basis of the time-course production of these products (Fig. 5). A gradual consumption of the fortified cardenolides started on 20 h after addition to the 2-day old cultures accompanying corresponding increase in their yields until complete consumption at the end of incubation term. Digoxin and digoxigenone seem to be the final

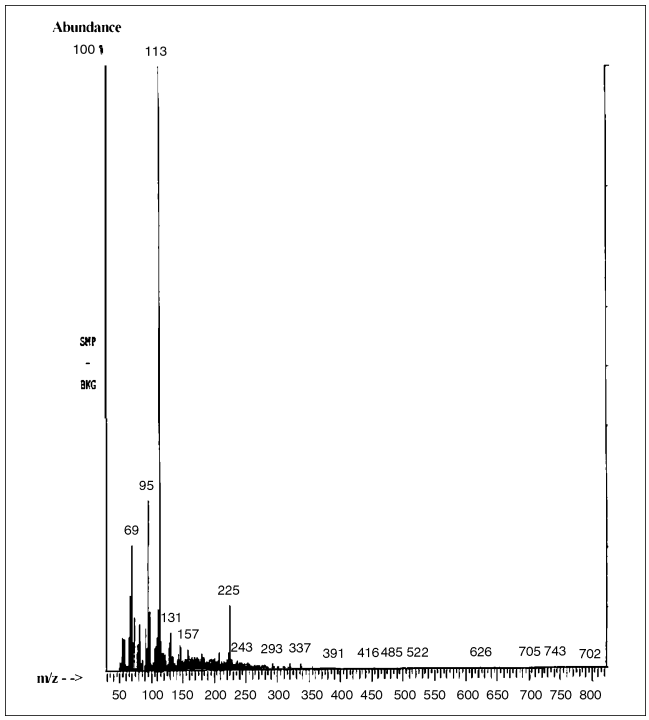
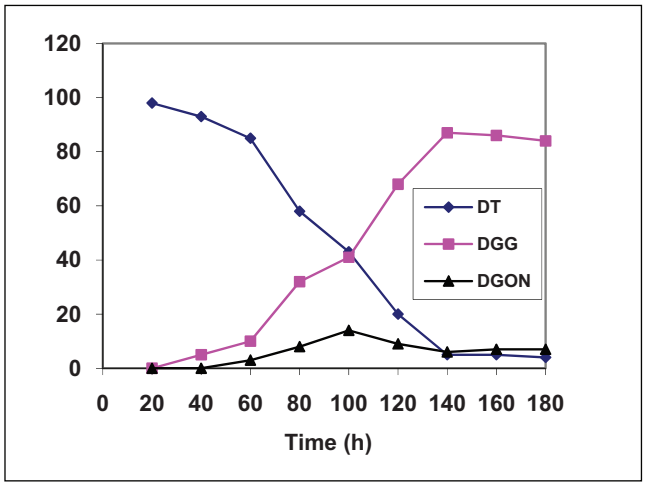
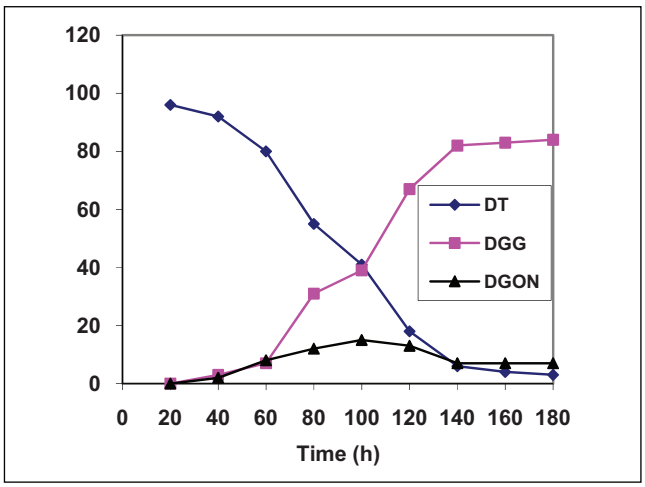


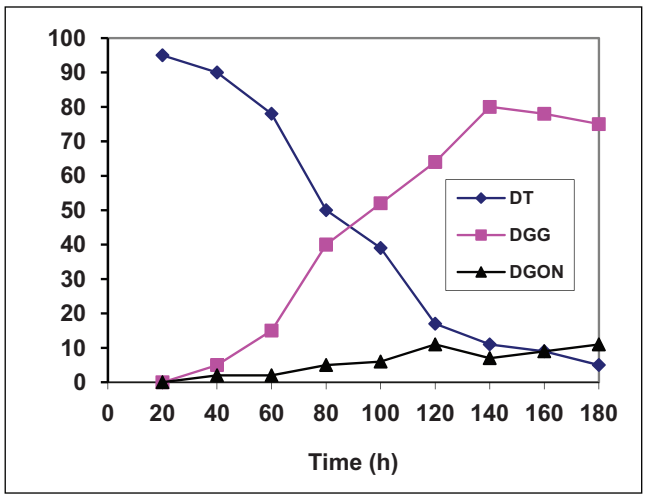
Fig. 4: Mass spectroscopy of biotransformed digoxin



(a)



(b)



(c)

Fig. 5: Biotransformation (%) vs time (h) of digitoxin (a), α-acetyl-digitoxin (b) and β-methyl-digitoxin (c) by Streptomyces EUSA2003B

transformation products evolved by 12β-hydroxylase activity harbored in the implicated organism (Table 3). The nature of the glycosidic side chains seems of minor interference in the yield of the bioconversion products since the productivity of digoxigenin from α-acetyl (Fig. 5b) and β-methyl-digitoxins (Fig. 5C) was a bit lower than that from DT (Fig. 5A). The successful transformation of digitoxins might partially be due to the use of cyclodextrin in the cultivation medium to increase permeability, and hence, facilitate its penetration through the cell walls of the *Streptomyces*. Studies of characterization, biochemical and physiological properties of selected Streptomyces strain EUSA-2003B revealed that morphologically, the spores of selected *Streptomyces* EUSA-2003B are tubular with spiky surface, and showing branching. Cultivation of selected *Streptomyces* strain on starch nitrate agar, glucose-nitrate, nutrient-agar and potato-agar media leads to the observation of variations in pigment secreted, aerial and substrate mycelia. The isolated *Streptomyces* EUSA-2003B possessed strongly coagulates and peptonizes skimmed milk and reduce nitrate, which indicate that *Streptomyces* EUSA-2003B possessed strong proteolytic and reducing activities. On the other hand, *Streptomyces* EUSA-2003B could moderately liquefy gelatin and failed decompose cellulose (Fig. 6).

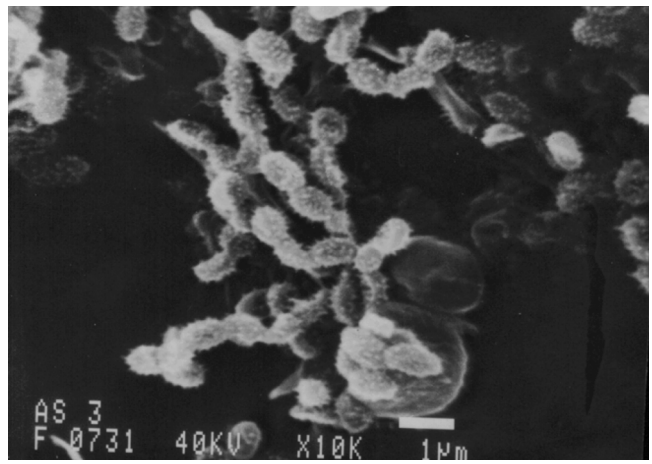


Fig. 6: Electron micrograph of the selected *Streptomyces* strain *BS-3* (X 10000)

The current study has demonstrated the capability of *Streptomyces* EUSA2003B to convert DT, α-acetyl and ß-methyl-DT into their corresponding 12ß-hydroxy derivatives in a 5-days fermentation process. The final yield (87%) of the therapeutically needed digoxin is reasonably high which gives this strain the advantage to be commercially feasible if submitted to further biotechnological applications.

3. Experimental

3.1. Instruments and chemicals

The UV absorption spectra were determined with PYE Unicon SP-800 and IR spectra were carried out using PYE Unicon SP-400. Mass spectroscopy was carried out with mass spectrophotometer at electron energy (70 mv) and final temperature 197°. The ^{1}H NMR, ^{13}C NMR, HMQC as well as ^{1}H^{1}H-COSY spectra were recorded using Bruker DRX-400 spectrometer (^{1}H400 MHz and ^{13}C 100 MHz) using TMS and internal standard for H and C nuclei. Chemical shift values (δ)are expressed in ppm while ***J*** coupling values are given in Hertz (Hz). Digitoxin, digoxin, digitoxigenin, digoxigenin, α-acetyl and β–methyl derivatives of DT and DG were supplied by Sigma Chemical co (USA). Solvents and other chemicals including those for HPLC and NMR spectroscopic methods are of high chromatographic grade (Aldrich, Human and Sigma Co.). Bi-distilled water was routinely used for purifications and water-containing solvent mixtures.

3.2. Cultivation of Digitalis lanata

Seeds of Digitalis lanata were purchased from European Authorized Agriculture Center, Germany. Seed were cultivated in October 2005. The seeds started to germinate after 15 days from cultivation and reached the beginning of the fruiting stage after 9 months. Then, the leaves were cut and rapidly dried in circulating hot air oven at 55–60 °C.

3.3. Microorganisms

Streptomyces strains were isolated in pure cultures from soil samples collected from different localities in Egypt (Al-Areesh, Northern Cost, and Cairo). Out of 153 isolates, a *Streptomyces* culture given the code EUSA-2003B possessed biotransformation capability during the preliminary screening program.

3.4. Cultures and cultivation conditions

Isolation and preservation of the *Streptomyces* isolates in pure cultures were carried on starch-nitrate medium contained (g.L^{-1} tap water): $NaNO_3$ (2.0), KH_2PO_4 (1.0), $MgSO_4.5H_2O$ (0.5) and 20 g of solubilized starch were eventually added (Yukari et al. 1996). *Difco* agar (2.2 g %) was melted and added whenever solid medium was needed and the *p*H was adjusted to 7.0 before sterilization (Waksman and Lechivalier 1961). For the screening and biotransformation experiments**,** the cultivation medium (CM) contained (g/L): yeast extract (3.5), soybean meal (5.0), KH_2PO_4 (5.0) and glucose (20.0) Tween 80 (0.15%) was finally added (Pádua et al. 2007).

3.5. Extraction of digitoxin from Digitalis lanata leaves

Extraction of digitoxin was carried out using Yukari et al. method (1996). In which, 25 ml of 50%methanol was added to 250 mg of leaf powder after ultrasonication for 1.5 h in an ice water bath the extract was filtered and then was dissolved in 3 ml of methanol then in 50 ml of ethyl acetate. Thin layer chromatographic (silica G_{254}) analysis was used according to the Carvalhas and Figueria method (1973) using chloroform-acetone-acetic acid (70: 30: 0.05) as eluting solvent. The method used for identification of digitoxin and biotransformed digoxin was according to Matthew et al (1971). Where, the mobile solvent methylene-methanol-formamide (80: 19: 1) and spray reagent, acidic ferric chloride were used.

3.6. Biotransformation experiments

Erlenmeyer flasks (250 ml) containing 75 ml of sterile starch-nitrate liquid medium were aseptically inoculated with fresh (7 day old culture) suspension of the *Streptomyces* isolate. Cardenolides (10 mg) were dissolved in DMF (1 ml) and added individually to 2-day growing cultures of the *Streptomyces* followed by further incubation period of 5 days under rotatory shaking (220 rpm) at 26–28 °C. Cyclodextrin (0.2%) was added to the DT-containing cultures to improve its permeability through the cell membrane of the organism. Control experiments containing medium plus *Streptomyces* isolate (C-1) and medium plus substrates (lacking the organism, C-2) were run alongside the biotransformation experiments. Immediately, at the end of incubation period (7 days), mycelia were filtered off, the biotransformation products were sequentially extracted with chloroform (3×50 ml) and chloroform/2-propanol (3:1,v/v) twice (80 ml each) using separating funnel on a cotton swab, solvent was vacuum removed at 52 °C, until residue. The obtained residues were combined, acid hydrolyzed (see below) and submitted to preparative RP-TLC (21) followed by preparative RP-HPLC (Kreis et al. 1990) where aqueous acetonitrile/water (37: 63) was used for eluting the products.

3.7. Acid hydrolysis of biotransformation products

Since the 12-**β**-hydroxylation of the aglycone moiety of cardenolides under investigation is the main target of the present study, we did not pay attention to any probable changes that might occur in the trisaccharide side chain (**α-**Methyl or **β**-acetyl-digitoxoses). Therefore, after extraction of the transformation products, they were subjected to acid hydrolysis (1N HCl at 55 °C for 35 min) to perform easier RP-HPLC detection of the aglycone residue of the transformed cardenolides.

3.8. Time-course monitoring of biotransformation

Under the abovementioned growth conditions, the capability of *Streptomyces* EUSA-2003B to hydroxylate the fortified cardenolides was monitored by removing portions (5 ml) of broth, aseptically, at regular time intervals (Table 2), extracted and acid hydrolyzed (only DT, **α**-Acetyl and **β**-Me derivatives). Aliquots of the extracted products were dissolved in 1 ml of MeOH:$CHCl_3$ (9:1, v/v) for the injection into Shimadzo HPLC apparatus and their retention times (R_t, Table 2) were measured. The characterization of the products was accomplished by comparing their R_t with authentic samples.

3.9. Characterization of the selected streptomyces EUSA-2003B

Numerous characterization of *Streptomyces* EUSA-2003B was carried out according to the method of Waksman (1967) that including morphological, cultural, biochemical characterization.

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