INFLUENCE OF INORGANIC IONS AND VITAMINS ON THE BIOTRANSFORMATION OF CHOLESTEROL TO ANDROSTENEDIONE BY *MYCOBACTERIUM* SP. MB-3683

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RESUMEN. Se investigó la influencia de algunos iones inorgánicos y vitaminas sobre la conversión microbiológica del colesterol con el empleo de la cepa *Mycobacterium* sp. MB-3683. El Mg^{2+} fue indispensable para los procesos de biotransformación y de crecimiento microbiano. La adición de Fe^{2+} estimuló la producción de 4-androsta-3,17-diona (AD), así como el crecimiento. El rendimiento de AD y el crecimiento microbiano no fueron estimulados significativamente por la adición de algunas vitaminas.

ABSTRACT. The influence of some inorganic ions and vitamins on the microbial conversion of cholesterol by *Mycobacterium* sp. MB-3683 was investigated. Mg^{2+} was indispensable for the biotransformation process and the microbial growth. The addition of Fe^{2+} stimulated the production of 4-androstene-3,17-dione (AD) and growth too. The yield of AD and the microbial growth did not appear to be stimulated significantly with the addition of some vitamins.

INTRODUCTION

The microbial steroid conversion process includes microbial growth, enzyme induction, the solubility of the steroidal substrate, the transformation of the substrate and extraction of products.[1] The efficiency of the fermentation is based on a sufficient yield, minimal formation of side products and avoidance of total degradation of the substrate.[2]

The first important aspect in the bioconversion of sterols is the microbial growth. The choice of medium ingredients balances the metabolism of the microorganisms and nature of the desired product.[3]

Szykula *et al.*[4] and Sedlaczek *et al.*[5] used a complex media to cultivate the *Mycobacterium* sp. MB-3683 mutant, but, the nutritional necessities of this strain have not been studied so far.

This paper descibes the influence of some ions and vitamins on the biotrasformation of cholesterol by *Mycobacterium* sp. MB-3683.

MATERIALS AND METHODS

Microorganism

Mycobacterium sp. MB-3683 degrades the sterol side chain to produce 4-androstene-3,17-dione (AD) as the major product, as described in a previous paper.[4]

Medium

Stock cultures were maintained on enriched nutrient agar consisting of 28 g nutrient agar (OXOID), 20g glycerol and 1 L distilled water. The culture medium (ENB) reported by Conner *et al.*[6] supplemented with glycerol (0.5 %) and Tween 80 (0.7 %), pH 7.0w as used as seed medium.

The basal medium for the production of AD consisted of 10 g glycerol; 5 g NaCl; 0,5 g K_2HPO_4;0, 5 gK H_2PO_4; 0,1 g $MgSO_4 \cdot 7H_2O$; 0,1 g $FeSO_4 \cdot 7H_2O$; 0,1 g $CaCl_2$;0, 05 g $MnSO_4 \cdot H_2O$ per liter of distilled water (pH 6,0). This synthetic medium was prepared with deionized water for the study of inorganic ions. For the influence of vitamins, these were added to the sterile synthetic medium under aseptic conditions in 20g $\cdot mL^{-1}$ each.

The cholesterol suspension was prepared as follows: 50mg of cholesterol (Merck), 1 mL of Tween 40s olution (15 %, w/v) and 4 mL of distilled water. This suspension was sterilized at 121 °C for 15 min and was added to 50mL of basal medium under aseptic conditions to give a final sterol and Tween 40 concentrations of 1 and 3 g $\cdot L^{-1}$ respectively.

Cultivation

The strain was grown in ENB medium for 48 h at 30 °C on an orbital shaker (200 r/min), then the culture was centrifuged at 7 000 r/min for 10 min, the pellet was washed with a sterile Tween 80 solution (0.7 %, w/v) and centrifuged again in similar conditions.

The biomass was resuspended in the sterile Tween 80 solution and homogenized by shaking. Five milliliters of this culture were inoculated into 50m L of a basal medium with cholesterol in a 300mL erlenmeyers flask. The cultures were incubated at 30 °C whith orbital shaking at 200 r/min for 6 d, then 5 mL of each culture were extracted. The cultures were sterilized at 121 °C for 30min .

Steroids determination

The culture broth was adjusted to pH 2,0w ith 3 mol $\cdot L^{-1}$-HCl and extracted four times with ethyl acetate (4 x 100 mL). The extracts were dried over anhydrous sodium sulphate and the solvent was evaporated "*in vacuo*". The AD levels from residues were measured by HPLC using a UV detector at 254 nm and a reverse phase column RP-18 (Merck), the mobile phase was water-methanol (35:65 v/v) pumped at 1,5 mL $\cdot$ min^{-1}.

Growth determination

The five milliliter culture extract was centrifuged in the conditions mentioned above and washed with the sterile distilled water. The pellet was suspended in 2 mL of 3 mol $\cdot L^{-1}$ NaOH solution and 1 mL of distilled water, then the samples were heated in a water bath at 100 °C for 10mi n . In the supernatant the protein content was measured by the Lowry method.[7]

Evaluation of experiments

The experiments were carried out by triplicate and the results were analysed by the tests ANOVA-1 and Duncan.[8]

RESULTS AND DISCUSION

Influence of the inorganic constituents

In considering the inorganic ions affecting the microbial conversion of sterols when the strain MB-3683 was grown on basal medium, certain ions seemed to be indispensable. In order to test this idea it was investigated the need for inorganic ions, the basal medium was modified by the omission and addition of the ion under investigation. The data were compared with yield obtained from incubation in the basal medium (control) from which the compounds under investigation were omitted or added. Throughout this study constant levels of carbon, nitrogen and phosphate sources were maintained.

The study of the effect of the omission of ions from the fermentation medium (Table I) showed that the production of AD was markedly reduced by the absence of Na^+ and Mg^{2+}, but the omission of the other ions studied did not affect the AD production.

TABLE I
Effect of the *omission* of the ions on the microbial conversion of cholesterol and strain growth

Variants	$Y_{p/s}$	Proteins
	(%)	($mg \cdot mL^{-1}$)
Control	38.9 (a)	0.38
NaCl	25.6 (b)	0.39
$FeSO_4$	37.5 (a)	0.42
$CaCl_2$	38.0 (a)	0.40
$MgSO_4$	9.6 (c)	0.18
$MnSO_4$	36.2 (a)	0.40

$Y_{p/s}$ Yield product/substrate added (milligrams of AD/milligrams of cholesterol added) x 100.
The values (b) and (c) are significantly different than four tests with (a) (test of Duncan $p \leq 0.05$).

Mg^{2+} seems to be the most important constituent of the medium. The function of this ion is related to the action of the enzyme system of the bioconversion process.[9] On the other hand, similar results were obtained by other authors when studied the 11α-hydroxylation of the progesterone by *R. nigricans*[10] and 6β-hydroxylation of different steroids by *A. niger*.[11] The levels of protein show that growth was significantly reduced by the magnesium salt absence, but the other ions did not affect the growth.

On the other hand, the omission of Fe^{2+},M n^{2+} and Ca^{2+} did not affect the AD production. This effect might be due to the fact that the presence of Mg^{2+} overlaps the action of the ferrous and calcium ions in the biotransformation process. For this reason, it was necessary to study the influence of the addition on each ion regardless of the culture medium.

The data presented in Table II show that the AD production was not stimulated by the additon of Na^{2+},C a^{2+} and Mn^{2+},a sl onga s Fe^{2+} and Mg^{2+} stimulated the biotransformation process, hence the yield product/substrate added ($Y_{p/s}$) obtained in presence of Mg^{2+} was similar to the control.

Only with the addition of ferrous and magnesium salts was the growth stimulated, but the levels of protein obtained in presence of these compounds were less than the control. Similar results had been reported by El Refai *et al.*[10] and Liu and Lee.[12]

All these results show that the magnesium ion is very important to produce AD and mycobacteria growth. For this reason, different concentrations of these ions were studied. The figure 1 shows that the production of AD was enhanced with the increase of the magnesium concentrations, and the maximal yield was obtained at 2.5 $\mu g \cdot mL^{-1}$ of Mg^{2+} which seemed to be optimal for the formation of AD. On the other hand, the growth was increased with the increase of the ion concentrations, and maximal growth was obtained at 29.6 $\mu g \cdot mL^{-1}$ of Mg^{2+}.

TABLE II
Effect of the *addition* of ions on the microbial conversion of cholesterol and strain growth

Variants	$Y_{p/s}$	Proteins
	(%)	($mg \cdot mL^{-1}$)
Control	38.9	0.38
NaCl	00	
$FeSO_4$	24.6	0.12
$CaCl_2$	00	
$MgSO_4$	37.8	0.26
$MnSO_4$	2.1	0

$Y_{p/s}$ Yield product/substrate added (milligrams of AD/milligrams of cholesterol added) x 100.

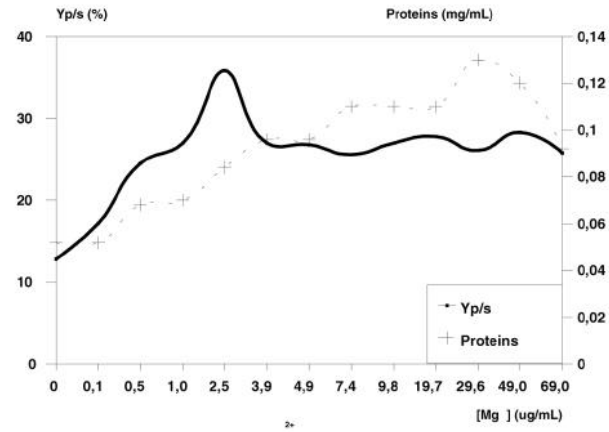

Fig. 1. Influence of magnesium concentration on the AD production and growth of the strain MB-3683.

Influence of vitamins

Addition to the medium of some individuals of the vitamin B group, such as panthotenic acid, thiamin, folic acid, biotin, nicotinic acid, riboflavin and other vitamins, such as ascorbic acid and myo-inositol proved to enhance the transformation reactions (Table III). The data obtained show that the production of AD was similar among the Control and the medium with inositol and ascorbic acid, the other vitamins studied did not stimulate the biotransformation process. An appreciable decrease in the level of AD was observed with riboflavin.

The protein levels show that growth was not affected or stimulated with the vitamins. Ratledge[13] reported that vitamins were not necessary to the growth of *Mycobacterium*. The results suggested that the vitamins were not indispensable in the fermentation broth to carry out the microbial conversion of cholesterol to AD using the strain *Mycobacterium sp*. MB-3683.

TABLE III
Effect of some vitamins on the microbial conversion of cholesterol and growth

Vitamins*	AD	Proteins
	($mg \cdot mL^{-1}$)	
Control**	287.7(ab)	0.31
Inositol	311.5 (a)	0.32
Ascorbic acid	280.6 (abc)	0.30
Panthotenic acid	272.4 (bcd)	0.32
Thiamine	263.5 (bcde)	0.32
Folic acid	259.8 (bcde)	0.32
Biotin	246.0(c de)	0.35
Nicotin acid	244.2 (de)	0.30
Riboflavin	235.6 (e)	0.31

* Vitamins were added to the sterile medium under aseptic conditions in 20 $\mu g \cdot mL^{-1}$ each.
** Control medium did not contain vitamins.

CONCLUSIONS

It was shown that magnesium ions are indispensable to produce AD from cholesterol and strain growth.

The iron ions stimulate the biotransformation and microbial growth processes.

It was demonstrated that the vitamins of β group, ascorbic acid and myo-inositol were not stimulated the biotransformation of cholesterol and strain growth, for that reason they were not indispensable in the fermentation broth.

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