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TRACKING THE EFFICIENCY OF CHOLESTEROL BIOCONVERSION BY *NOCARDIA* SP. MTCC 1534 IN PRESENCE OF SOME RING CLEAVAGE INHIBITORS BY GAS CHROMATOGRAPHY

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ABSTRACT

Nocardia sp. MTCC 1534 was found to utilize cholesterol as sole source of carbon and energy. Growing culture of the organism was incubated with reported 9-alpha-hydroxylase inhibitors for accumulation of 17-ketosteroids. The inhibitors used in the present investigations were isopropanol, cupric chloride and 8-hydroxy quinoline. The effect of concentration and time of addition of inhibitors was studied and products accumulated were analysed with the help of thin layer chromatography. The dried extracts of bioconversion medium in organic solvents were subjected to Gas chromatography and peaks of bioconversion products analysed by Mass spectroscopy. In case of *Nocardia* sp. MTCC 1534 it was observed that all 3 inhibitors i.e. cupric chloride, 8-OH-quinoline and isopropanol give maximum variety of products at 48 hrs of incubation periods which decline afterwards. Hence, 48 hr incubation was recommended time period after growth of organisms and substrate addition for accumulation of products which were proposed to be 17-ketosteroids on the basis of mass analysis.

KEYWORDS: **cholesterol, steroid bioconversion 17-ketosteroids, cholesterol side chain cleavage, 9-α-hydroxylase inhibitors.**

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INTRODUCTION

The production of steroid drugs and hormone is one of the best examples of the successful application of microbial technology in large scale industrial processes. Steroids constitute a particular class of lipids characterized by a typical tetra cyclic skeleton, composed of one five-membered ring and three fused six membered rings. Steroids are widely used as therapeutic agents and since their inception in the market, research efforts have been made in order to improve production processes as well as to develop novel synthetic molecules, which enhanced efficiency and reduced side effects. It has been reported that the metabolic pathway of cholesterol degradation by bacteria has not been completely established [1]. Several possible intermediates have not been identified and many pathway delineations have not involved the use of the cholesterol molecule and just one bacterial species. A screening of various microbial strains for side chain cleavage of cholesterol was attempted. The main aim of microbial cholesterol side chain cleavage is to produce 17-ketosteroids by bioconversion which can be converted into many pharmacologically active compounds. Several microbial bioconversions of steroids and sterols have been reported. The identification of the 11α-hydroxylation activity of a *Rhizopus* species was found and it was a decisive step in the development of the practical synthesis of steroids with useful biological activity [2]. The production of testosterone from cholesterol using a single strain, either *Mycobacterium* sp. NRRL B-3805 or *Lactobacillus bulgaricus* sp. has also been reported [3]. A *Nocardia* sp. soil isolate could convert cholesterol to pregn-4-en-3-oxo-20-carboxylic acid, androst-4-en-3,17-dione (AD), and androsta-1,4-dien-3,17-dione (ADD) in the presence of 17-hydroxyquinoline, focusing mainly on steroid hydroxylation, dehydrogenation and sterol side-chain cleavage[4]. These biotransformations, mostly associated to chemical synthesis steps, have provided adequate tools for the large scale production of natural or modified steroid analogues [5]. The use of inhibitors to enable detection of intermediary metabolites was utilized [6]. The use of 9-alpha-hydroxylase inhibitors during cholesterol bioconversions is recommended for accumulation of 17-ketosteroids.

MATERIALS AND METHODS

Cholesterol was purchased from Sigma chemicals. All reference steroids were 100% pure as judged by thin layer chromatography. The general chemicals & media components required for the study were purchased from High media, Merc & Loba.

Microorganisms

The following strain of microorganism was purchased from MTCC, Institute of Microbial Technology and Chandigadh, India.

1) *Nocardia* sp. MTCC 1534

The strain was maintained on nutrient agar slants with appropriate inducer steroid (1mg/l) and stored in a refrigerator at 4˚C.

Bioconversion by growing cells:

Nutrient broth media was prepared & pH adjusted to 7 with the help of 0.1 N NaOH or HCl. Medium (20 ml) was dispensed in a series of 100 ml conical flasks, sterilized at 121ºC for 15 minutes & allowed to cool to room temperature. The flasks were inoculated with actively growing culture of the strain of microorganism in the same medium, allowed to grow overnight at 37˚C on shaking incubator at 100 rpm & 2 mg cholesterol dissolved in 0.2 ml acetone was added. Inhibitor was added after 24 hrs of addition of substrate addition. 8-hydroxy quinoline was dissolved in acetone & copper chloride in water while isopropanol was added as such in the liquid form.

Extraction of the sample steroid:

Samples(1ml) were drawn at specific intervals from bioconversion flasks & extracted twice with equal volume of ethyl acetate. Solvent was evaporated by keeping the tubes in boiling water bath. The test tubes were taken from boiling water bath & concentrated

samples were loaded on TLC plates which was followed by GC analysis of Bioconversion broth and MASS analysis of selected bioconversion products

RESULTS AND DISCUSSION

Gas chromatography has been routinely employed for analysis of bioconversion products during steroid bioconversions. The most precise procedure for detection of unknown substances is a combination of GC and MS. Gas chromatography/mass spectrometry is a two-step process, where GC separates the sample into its constituent parts, while MS provides the exact molecular identification of the compounds. The method is approximately 100 to 1,000 times more sensitive than TLC. Gas chromatography has been successfully employed for monitoring the progress of 1,2-dehydrogenation of androstenedione by *Fusarium oxysporum* [7]. Sequential conversion of cortexolone to prednisolone by immobilized mycelia *Curvularia lunata* and immobilized cells of *A. simplex* have been reported where researchers used gas chromatography for the steroid analysis [8].

Gas Chromatography analysis of bioconversion products

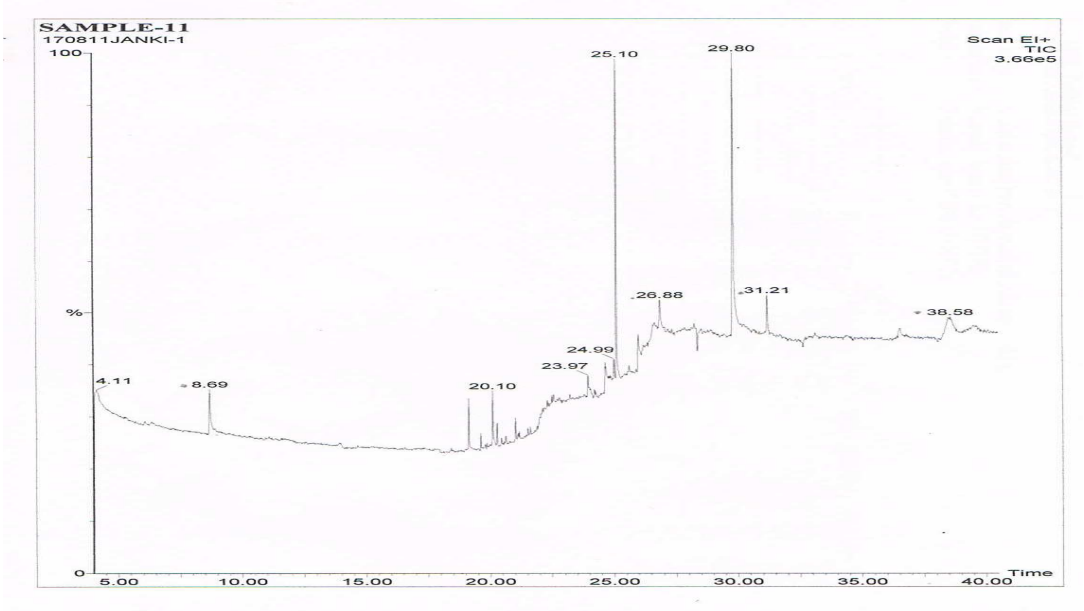

Figure 1a

Gas chromatogram of cholesterol bioconversion product of Nocardia sp. MTCC 1534 in presence of $CuCl_2$ at 48 hrs of incubation.

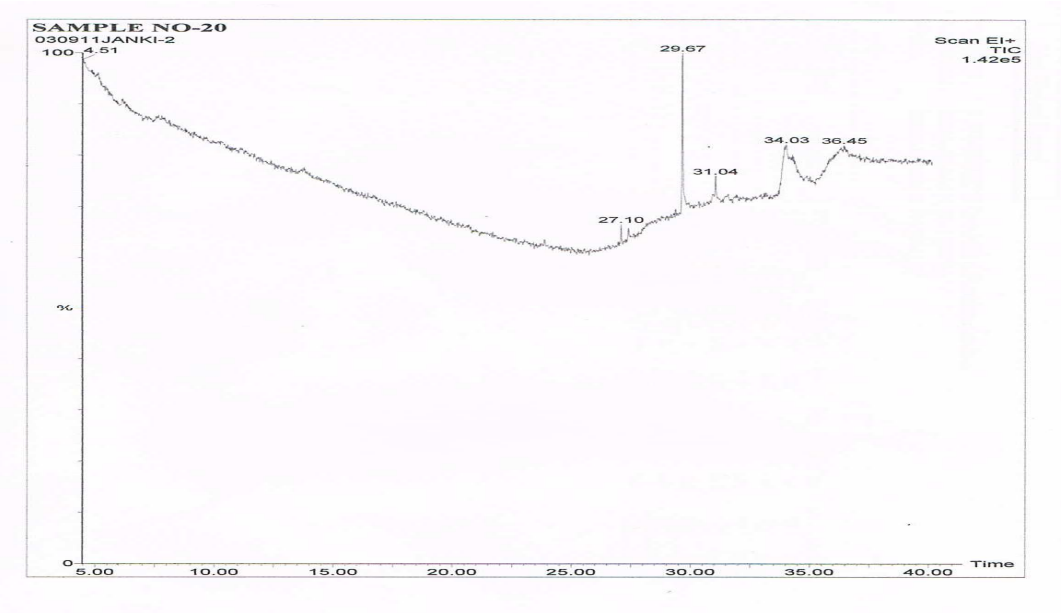

Figure 1b

Gas chromatogram of cholesterol bioconversion product of Nocardia sp. MTCC 1534 in presence of $CuCl_2$ at 120 hrs of incubation.

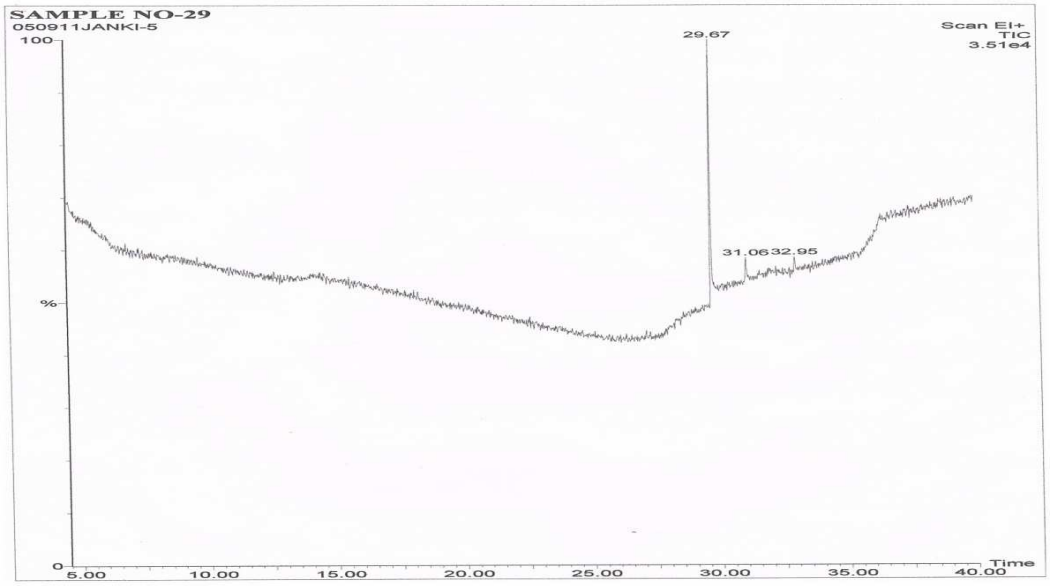

Figure 1c

Gas chromatogram of cholesterol bioconversion product of Nocardia sp. MTCC 1534 in presence of $CuCl_2$ at 168 hrs of incubation.

It is evident from figure 1(a, b, c) that when $CuCl_2$ is used as an inhibitor with *Nocardia* sp. MTCC 1534 during cholesterol bioconversion, maximum variety of products are seen at 48 hrs of incubation which disappear afterwards. From above all we can see that 48 hrs is the best time period for the product formation.

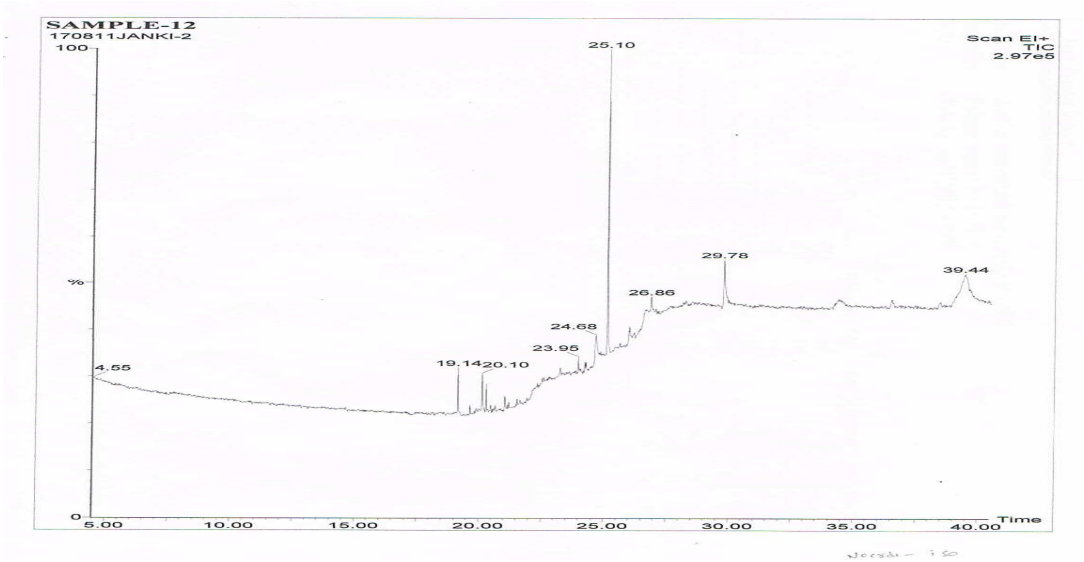

Figure 2a

Gas chromatogram of cholesterol bioconversion product of Nocardia sp. MTCC 1534 in presence of isopropanol at 48 hrs of incubation.

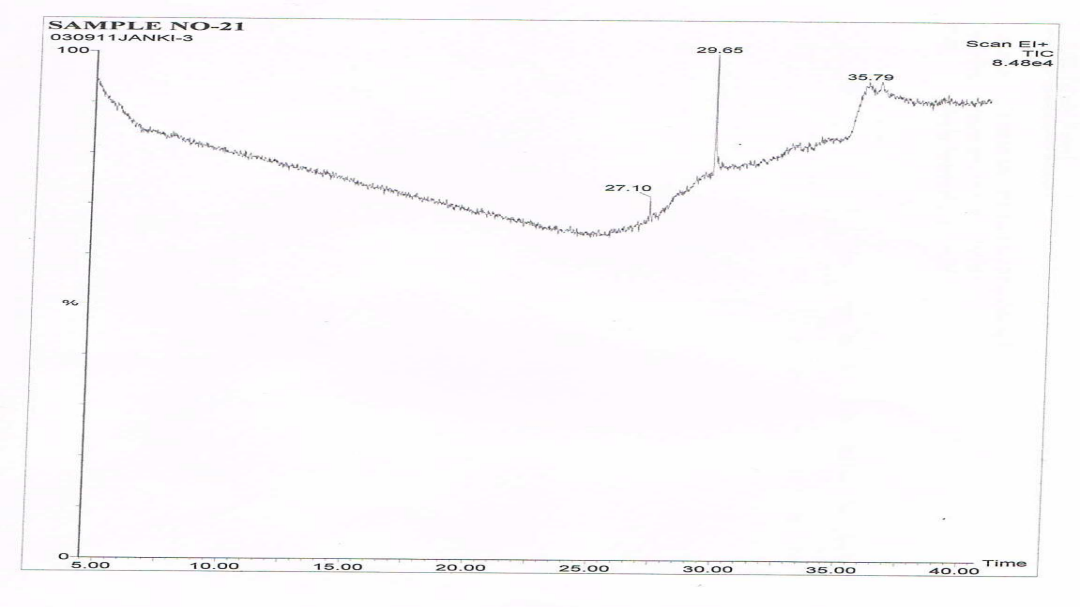

Figure 2b

Gas chromatogram of cholesterol bioconversion product of Nocardia sp. MTCC 1534 in presence of isopropanol at 120 hrs of incubation.

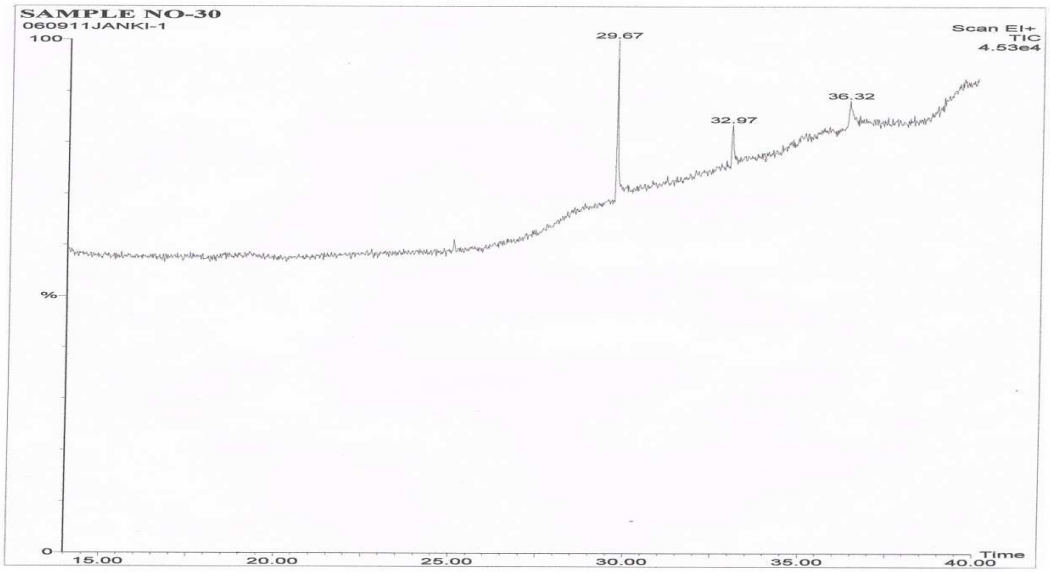

Figure 2c

Gas chromatogram of cholesterol bioconversion product of Nocardia sp. MTCC 1534 in presence of isopropanol at 168 hrs of incubation.

It is evident from figure 2(a, b, c) that when isopropanol is used as an inhibitor with *Nocardia* sp. MTCC 1534 during cholesterol bioconversion, maximum variety of products are seen at 48 hrs incubation which declines afterwards.

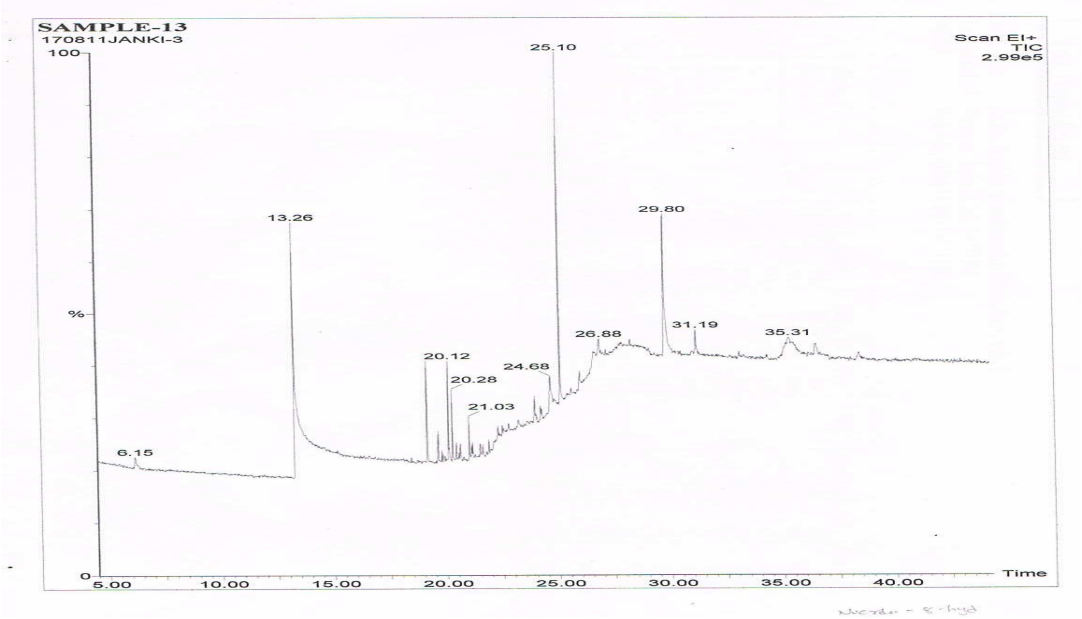

Figure 3a

Gas chromatogram of cholesterol bioconversion product of Nocardia sp. MTCC 1534 in presence of 8-OH-quinoline at 48 hrs of incubation.

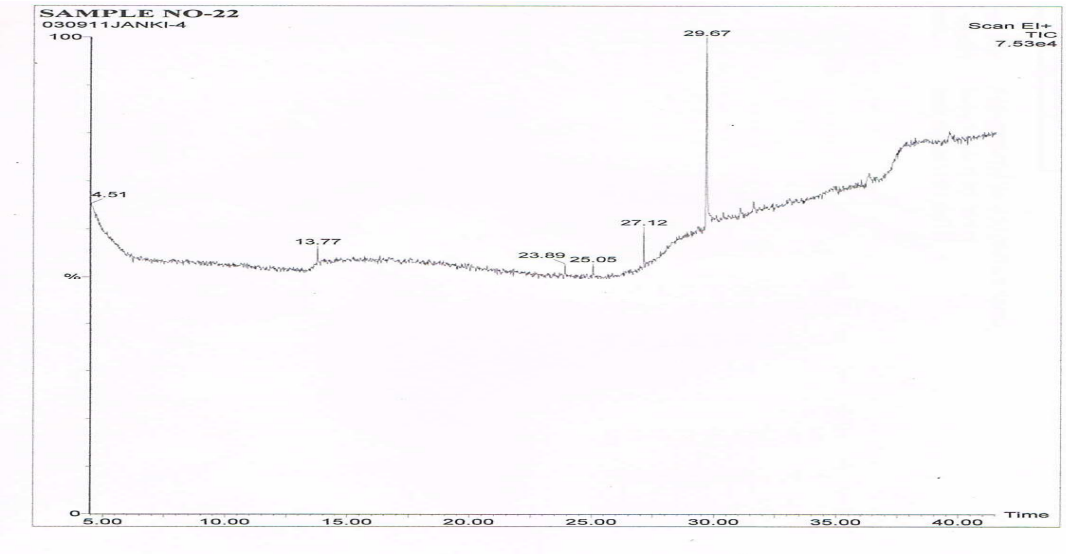

Figure 3b

Gas chromatogram of cholesterol bioconversion product of Nocardia sp. MTCC 1534 in presence of 8-OH-quinoline at 120 hrs of incubation.

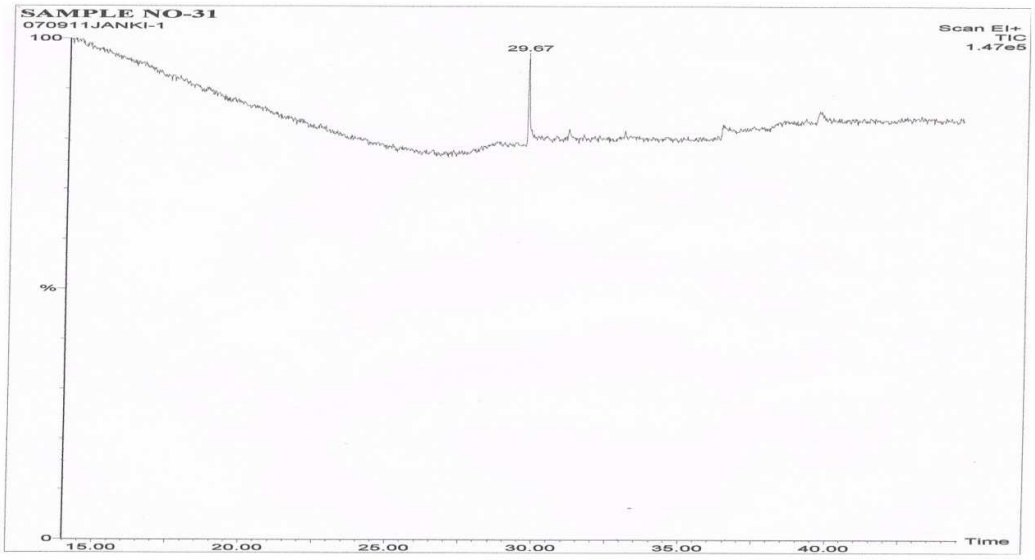

Figure 3c

Gas chromatogram of cholesterol bioconversion product of Nocardia sp. MTCC 1534 in presence of 8-OH-quinoline at 168 hrs of incubation.

It is evident from figure 3(a, b, c) that when 8-OH-quinoline is used as an inhibitor with *Nocardia* sp. MTCC 1534 during cholesterol bioconversion, maximum variety of products are also seen at 48 hrs incubation which declines afterwards.

It is obvious from gas chromatographic analysis that cholesterol is efficiently degraded by *Nocardia* sp. however, accumulation of intermediate steroids depends on a proper selection of inhibitor, it's concentration, time of addition and incubation time allowed for fermentation. The data highlights accumulation of certain stable intermediates under above mentioned specified conditions. An attempt was done to identify the nature of the compounds on the basis of their mass analysis and comparison with available literature. IR and NMR analysis of the compounds was not feasible taking into

account their low concentration in the bioconversion medium extracts.

The spectral analysis of mass spectrum of peak 8.69 of gas chromatographic analysis of cholesterol by *Nocardia* sp. MTCC 1534 with $CuCl_2$ as inhibitor after 48 hrs incubation (Figure 1a) indicates lack of steroid ring and hence it was concluded to be a non-steroidal product.

Mass spectrum of peak 26.88 of gas chromatographic analysis of cholesterol by *Nocardia* sp. MTCC 1534 with $CuCl_2$ as inhibitor after 48 hrs incubation (Figure 1a) shows mass ions corresponding to 284 and 283 molecular weight indicating that the compound must be a 17-keto steroid. The steroidal nature of the compound is supported by significant m/e 119. GC analysis shows the compound is less abundant although stably accumulated with *Nocardia* sp. MTCC 1534 organisms in presence of $CuCl_2$, 8-OH-quinoline and isopropanol.

Mass spectrum of peak 31.20 of gas chromatographic analysis of cholesterol by *Nocardia* sp. MTCC 1534 with $CuCl_2$ as inhibitor after 48 hrs incubation (Figure 1a) shows mass profile in the range of 17-ketosteroids. Potent steroid ring structure is indicated by m/e 124, particularly 4-en-3-one [1]. Further using the same reference lack of important peaks at 271 indicates absence of side chain. Though the GC analysis shows low concentration of this product during cholesterol bioconversion by *Nocardia* sp. MTCC 1534 in presence of $CuCl_2$ (Figure 1a), there is sufficient scope of improving the concentration by proper parameter optimization (Figure 1a). Highlights slow degradation of cholesterol suggesting positive possibilities of the above mentioned scope.

It can be concluded on the basis of GC and mass analysis that *Nocardia* sp. MTCC 1534 in presence of $CuCl_2$ is one of the important candidates to be selected for optimal accumulation of 17-ketosteroid intermediates.

Mass spectrum of peak 23.94 of gas chromatographic analysis of cholesterol by *Nocardia* sp. MTCC 1534 with isopropanol as inhibitor after 48 hrs incubation, mass spectrum of peak 25.10 of gas chromatographic analysis of cholesterol by *Nocardia* sp. MTCC 1534 with isopropanol as inhibitor after 48 hrs incubation and mass spectrum of peak 13.25 of gas chromatographic analysis of cholesterol by *Nocardia* sp. MTCC 1534 with 8-OH-quinoline as inhibitor after 48 hrs incubation(Figure 2a) suggest non- steroidal nature of the compounds.

Mass spectrum of peak 31.19 of gas chromatographic analysis of cholesterol by *Nocardia* sp. MTCC 1534 with 8-OH-quinoline as inhibitor after 48 hrs incubation (Figure 3a) suggests 4-en-3-one structure (m/e 124, 100% abundance). It also suggests 17-keto side chain and correlation with GC analysis highlights *Nocardia* sp. MTCC 1534 for accumulation of this compound in presence of 8-OH-quinoline.

CONCLUSION

In case of *Nocardia* sp. MTCC 1534 it was concluded that all 3 inhibitors i.e. $CuCl_2$, 8-OH-quinoline and isopropanol give maximum variety of products at 48 hrs of incubation periods which decline afterwards. An attempt was done to identify the nature of the compounds on the basis of their mass analysis and that data led to conclusions that some compounds were steroidal in nature while others were non-steroidal compounds. *Nocardia* sp. MTCC 1534 with $CuCl_2$ is one of the important candidates to be selected for the accumulation of the 17-ketosteroids.

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REFERENCE

1. Owen R. W., Arthur N-Mason A. and Bilton R. F., The degradation of cholesterol by P.Putida sp. NCBI 10590 under aerobic condition. Journal of lipid research, 24: 1500-1511, (1983).
2. Hogg J.A., steroids, the steroid community, and Upjohn in perspective: a profile of innovation. Steroids, 257: 593-616, (1992).
3. Kumar R., Dahiya JS., Singh D and Nigam P., Bioresour Technol, 78(2): 209-11, (2001).
4. Whitmarsh J.M., Intermediates of microbiological metabolism of cholesterol. Biochem. J, 9: 1-23, (1964).
5. Mahato SB and Mukherjee., A. Steroid transformation by microorganisms. Phytochemistry, 23: 2131-54, (1984).
6. Arima K., Nagasawa M., Bae M. and Tamura G., Microbial transformation of sterols, Part I. Decomposition of cholesterol by microorganisms. Agricultural and Biological Chemistry, 33: 1636–1643, (1969).
7. Vincke B. J., Devleeschouwer M.J., Dony J. and Patriarche G.J., Efficiency of bioconversion of steroids by *Fusarium oxysporum* into Δ1,4-androstadiene-3,17-dione using gas chromatography. International journal of pharmaceutics, 32 (1): 61-69, (1986).
8. Mahato S. B. and Majomdar I., Current trends in Microbial Steroid Transformation. Phytochemistry, 4: 297-316, (1985).