MICROBIOLOGICAL TRANSFORMATION OF 3-METHOXY-8, 14-SECO-1,3,5(10),9(11)-ESTRATETRAEN-14, 17-DIONE TO ITS 17β-HYDROXY DERIVATIVE

VINOD BIHARI, A. W. KHAN and V. C. VORA
Fermentation Technology Division, Central Drug Research Institute, Lucknow 226 001, *India.*

ABSTRACT

Optimum conditions for obtaining the desired transformed product 17β-hydroxy secol from seco dione using Pichia farinosa Y-118 have been reported. Shake flask and small glass bioreactor results could be transferred to laboratory scale fermenter on the basis of volumetric oxygen transfer coefficient ($K_L a$) measurement.

INTRODUCTION

In the chemical synthesis of estrone and its analogues,3-methoxy-8, 14-seco-1,3,5(10), 9(11)-estratetraen-14, 17-dione (secodione) is obtained by converting 6-methoxytetralone to tetralole using Grignards reaction followed by condensation with methyl cyclopentane dione. This secodione is stereospecifically reduced to its 17β-hydroxyl derivative, before cyclisation, to obtain the required optically active isomer. This reduction, when carried out chemically, yields a mixture of α and β isomers. After Mamoli and Vercellone[1] first reported the microbiological reduction of 17-oxo steroids to 17β-hydroxy steroids, Gibian *et al*[2], Kosmol *et al*[3], Szentrimai *et al*[4] and Gulaya *et al*[5] have reported the stereospecific reduction of secodione to its 17β-hydroxy derivative (secol) using yeast cultures. Szentrimai *et al*[4] reported the presence of two hydroxysteroid oxidoreductases in *S. uvarum*. One reduces the 17-keto to 17β-hydroxyl while the other reduces 14-keto to 14α-hydroxyl function. They also demonstrated that the reduction of 14-keto could be selectively inhibited by β-naphthol. Buzby *et al*[6] have also reported this transformation using strains of *Pichia farinosa*. The transformation reaction is shown in figure 1.

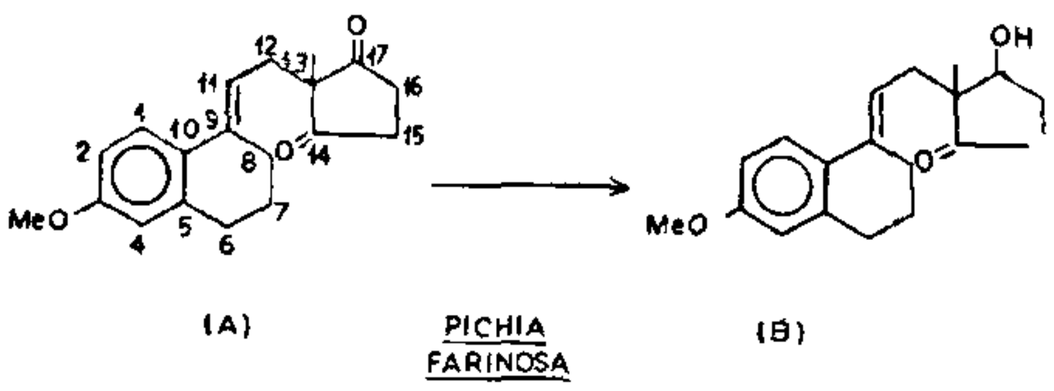

Figure 1. Microbial transformation of 3-methoxy-8, 14-seco-1,3,5(10),9(11)-estratetraen-14,17-dione (A) to its 17β-hydroxy derivative (B).

MATERIALS AND METHODS

Culture: Pichia farinosa NRRL Y-118 was used in this study. The studies presented here have been carried out with the best colony isolated by natural selection from the type culture presently used. The culture was maintained on a medium containing (g/l): yeast extract, 3; malt extract, 2; peptone, 5; glucose, 10 and agar, 20.

Transformation: The culture was grown in a medium containing (g/l): cornsteep liquor, 20; dextrose, 40; and malt extract, 1. The pH of the medium was adjusted to 6.7. Culture was incubated on a rotary shaker for 20–24 hr at 28° ± 1°C before the required quantity of "Secodione" dissolved in 2 ml of an organic solvent (ethanol, N,N′-dimethyl formamide or dimethyl sulphoxide) was added and the incubation continued for further 40 hr.

Isolation, purification and identification of the product: Cells and supernatant obtained by centrifugation of the reaction mixture were separately extracted with acetone and chloroform respectively. The two extracts were concentrated and combined to give a crude product in the form of thick syrupy mass. The product was crystallized in heptane solution. Identity of the product was confirmed by its m.p. (112°C), specific rotation ($\alpha_D = -39°$ in dioxane), UV absorption (264 nm in methanol), infra-red and proton magnetic resonance spectra.

Quantitative estimation: Modified Zimmermann reaction as reported by Pontius[7] was used for quantitative estimation of 'secol'. The substrate and product were separated on silica gel GF_{254} plates in developing solvent chloroform : ether (10 : 1). Equal areas of adsorbent containing secol and blank adsorbent were scraped and extracted with methanol, the solvent was

evaporated and the colour developed. Known amounts of pure secol were also chromatographed along with the reaction mixture, as a reference, at three concentrations. The amount of 'secol' in the reaction mixture was so adjusted that it was within the three concentrations of standard. All estimations were carried out in triplicate.

RESULTS AND DISCUSSION

Out of the various studies carried out the optimum values obtained for some of the common parameters were as follows: (i) pH = 6 to 7, (ii) temperature = 28° ± 1°C, (iii) optimum growth period before substrate addition = 20 hr, (iv) reaction period after substrate addition = 35 hr, (v) glucose concentration = 4 to 5 g/l.

The pH of the broth which was 6.5 at the time of addition of substrate, steadily dropped to 3.8 at the end of 40 hr of transformation reaction. Maintaining the pH at 6.5 was found to have a deleterious effect on the transformation. Secodione was added to the growing cultures of different ages from 10 hr onwards and the maximum conversions obtained were recorded. It was observed that after 20 hr of growth the culture had become suitable to obtain the best conversions. During further transformation after substrate addition, no substrate was transformed in the first 10 hr period; it was only after that, the peak level of cell activity was observed which remained at that level upto 30 hr before declining. This is reasonable evidence to assume that the enzyme system involved is inducible in nature and it takes about 10 hr before product formation is observed in the system studied. Glucose concentration is often critical in enzymatic reactions involving inducible enzyme systems. Increased activity was observed upto 4 and 5 g/l glucose concentrations and at 6 g/l the activity level started declining showing repression of the enzyme system involved.

Effect of substrate concentration: As the substrate concentration was increased from 1 mg/ml to 10 mg/ml the per cent conversion fell steadily. However, as shown in figure 2, the reaction rate increased with increase in substrate concentration till it reached a maximum at 5 to 6 mg/ml substrate concentration.

Effect of β-naphthol: As mentioned before β-naphthol selectively inhibited one of the two hydroxysteroid

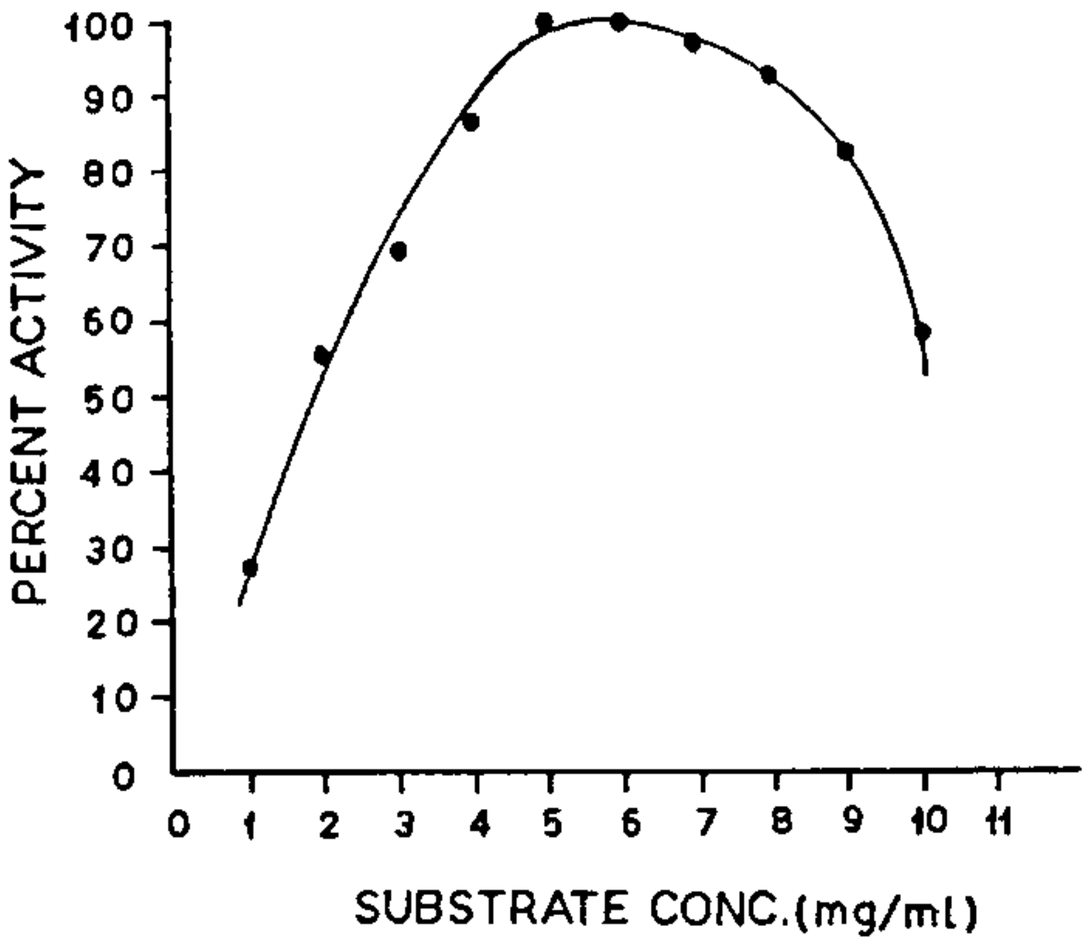

Figure 2. Effect of substrate concentration.

oxidoreductases in *S. uvarum* responsible for the reduction of 14-keto to 14α-hydroxyl function in the 'secodione'. Neither any other transformed product nor any effect was observed on the yields of desired '17β-secol' indicating that the other enzyme is possibly not present in this strain of *P. farinosa.*

Oxygen transfer: All the initial experiments reported above were carried out in shake flasks. To scale up the process a 500 ml, all glass bio-reactor fitted with baffles, magnetic stirrer and air sparger tube was used. In 100 ml of the medium in this reactor, aeration and agitation were varied till the results of growth and transformation obtained were the same as those from shake-flask experiments. To determine the agitation and aeration conditions prevailing in this 500 ml reactor, the sulphite oxidation method[8] was used. This method of measurement of volumetric oxygen transfer coefficient (K_La) does not represent the actual conditions inside a fermentor where microbial transformation is taking place; however, it does help in determining the agitation and aeration conditions prevailing in vessels of different sizes and geometry to obtain desired degree of mixing and does, to a certain extent, help in scale up. To determine K_La in the 500 ml reactor 100 ml of sulphite solution was taken and the same agitation and aeration conditions were used as those employed in the actual transformation. This way the K_La was found to be 300 hr^{-1}. Then, in a 5 litre New Brunswick fermentor with 3 litre working volume, the K_La values were determined. They were

180 hr^{-1} with 1 vvm aeration and 300 rpm agitation and 360 hr^{-1} with 1 vvm aeration and 400 rpm agitation. Where the K_La was 360 hr^{-1} the yields of transformed product were the same as those obtained in the 500 ml reactor, but they fell to 67% of maximum at the lower K_La value.

The quantitative conversion assayed by TLC and colorimetry was found to be 75%, however, recovery of the pure product was 80% by the method reported above. The remaining product and unreacted substrate could then be separated by silica gel column chromatography using benzene and benzene-methanol mixture as the eluting solvent.

Using identical conditions of transformation similar yields of 17β-hydroxylated product of 13-ethyl-3-methoxy-8, 14-seco-gona-1,3,5(10), 9(11)-tetraen-14-17-dione were also obtained.

The importance of estrone derivatives has increased in the family welfare programs. With the stereospecific reduction of the 17-keto group to 17β-hydroxyl of either 3-methoxy-8, 14-seco-1,3,5(10), 9(11)-estratetraen-14, 17-dione or of 13-ethyl-3-methoxy-8, 14-seco-gona-1,3,5(10), 9(11)-tetraen-14, 17-dione, the total synthesis of estrone and its analogues has become possible.

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ANNOUNCEMENT

INDIAN NATIONAL SCIENCE ACADEMY, NEW DELHI, YOUNG SCIENTISTS AWARD

The following received the 'Young Scientists Award': 1. Dr P. K. Agarwal, Indian Agricultural Research Institute, New Delhi, 2. Dr Ram Boojh, Department of Environment, Utter Pradesh, 3. Dr Deepak Dhar, Tata Institute of Fundamental Research, Bombay, 4. Dr (Mrs.) Chandana Mishra, Banaras Hindu University, Varanasi, 5. Dr (Mrs.) Sumitha Jha, Botany Department, Calcutta University, Calcutta, 6. Dr H. R. Krishnamurthy, Indian Institute of Science, Bangalore, 7. Dr (Miss) Virendra Jeet Lamba, Delhi University, Delhi, 8. Dr Michael Lobo, Indian Institute of Science, Bangalore, 9. Dr G. Malakondiah, Defence Metallurgical Research Laboratory, Hyderabad, 10. Dr B. V. Venkatarama Prasad, Indian Institute of Science, Bangalore, 11. Dr H. V. Ram Babu, Osmania University, Hyderabad, 12. Dr Narayanachandra Rana, Tata Institute of Fundamental Research, Bombay, 13. Dr D. D. Sharma, Indian Institute of Science, Bangalore, 14. Dr D. S. Sastri, International Crop Research Institute for Semi-Arid Tropics, Hyderabad, 15. Dr A. K. Saxena, Central Drug Research Institute, Lucknow, 16. Dr A. K. Srivastava, Gorakhpur University, Gorakhpur, 17. Mr. Rakesh, Bhabha Atomic Research Centre, Bombay.