

SOLASODINE STEROID BIOCONVERSION BY MYCOBACTERIUM PHLEI DSM 43286.

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ABSTRACT

Bioconversion of solasodine by *Mycobacterium phlei* DSM 43286 was conducted to obtain intermediate compounds which might be used as precursor in the production of steroidal drugs, i.e. androst-4-en-3, 17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD).

M. phlei was firstly grown in nutrient broth medium at 37 °C for 8.5 hours with agitation of 200 rpm. The bacterial culture thus obtained was used as starter to inoculate the conversion medium containing 0,02% solasodine as the substrate and 0.01% 8-hydroxyquinoline as inhibitor. Bioconversion was conducted for 12 days at 37 °C using the same speed of agitation. Analysis of the bioconversion products was carried out using samples taken periodically at a 24-hour interval by TLC and HPLC methods.

TLC analysis using chloroform-ethyl acetate (80:20) as eluent, measurement of the maximum wavelength and molar extinction coefficient value showed that AD and ADD was not found in the fermentation products, but other intermediate compound might be present. However, HPLC analysis of the fermentation products using μ -porasil column and benzene-ethylacetate-chloroform (40:80:10) as eluent, showed peaks with retention time similar to that of AD (during the 2nd – 9th day of incubation) and, ADD (during the 5th – 6th day of fermentation) and, other unknown peaks.

INTISARI

Biokonversi solasodin oleh *Mycobacterium phlei* DSM 43286 dilakukan untuk mendapatkan suatu senyawa intermediet yang merupakan prekursor pada pembuatan obat-obat steroid seperti Androst-4-en-3,17-dion (AD) dan Androsta-1,4-dien-3,17-dion (ADD).

M. phlei ditumbuhkan dalam medium cair nutrisi pada suhu 37 °C selama 8,5 jam dengan guncangan orbital 200 rpm. Selanjutnya suspensi *M. phlei* ini diinokulasikan ke dalam medium konversi yang mengandung 0,02% solasodin dan 0,01% 8-hidroksiakuinolin sebagai inhibitor. Biokonversi dilakukan pada suhu 37 °C selama 12 hari dengan guncangan orbital yang sama. Analisa hasil biokonversi dilakukan setiap interval 24 jam menggunakan metoda kromatografi lapisan tipis (KLT) dan kromatografi cairan kinerja tinggi (KCKT).

Analisa KLT menggunakan eluen kloroform-etil asetat (80:20), pengukuran panjang gelombang maksimum dan harga koefisien ekstingsi molar menunjukkan bahwa AD dan ADD tidak terdapat dalam hasil fermentasi tetapi kemungkinan ada senyawa-senyawa intermediet yang lain. Namun demikian, analisa KCKT dari campuran hasil fermentasi menggunakan kolom μ -porasil dan campuran benzen-etil asetat-kloroform (40:80:10) sebagai eluen memperlihatkan adanya puncak dengan waktu retensi yang hampir sama dengan AD (selama hari ke-2 sampai hari ke-9 waktu inkubasi), ADD (selama waktu fermentasi hari ke-5 sampai dengan hari ke-6) dan puncak-puncak lain yang tidak diketahui.

INTRODUCTION

Steroid compounds are widely used in medicine as drugs, vitamins and hormones as well as for contraception. The global demand for steroid compounds is increasing mainly for production of contraceptive drugs. To meet the high demand for contraceptive drugs, Indonesia has imported a large amount of steroid compounds annually in the last few years. Therefore, effort is needed to find ways in producing the desired compounds economically.

Some plants belong to the genera of *Dioscorea*, *Trigonella*, *Agave*, *Costus* and *Solanum* have been used as raw materials for production of contraceptive drugs since they contain steroid precursors such as hexogenin, diosgenin and solasodine (1). The decrease in diosgenin supply in the world has stimulated the search for new sources of steroid precursors. Solasodine is probably the most appropriate in replacing diosgenin, since it has very similar chemical structure with the latter (Figure 1). *Solanum khasianum* spp is a potential solasodine source and easily grown in Indonesia (1). The berries contain around 3% (dry weight) solasodine.

Conversion of steroid to produce important intermediate compounds needed in the synthesis of contraceptive drugs may be accomplished by microbiological method. From the various contraception methods practiced in Indonesia, the use of oral drugs is one of the popular method, and the government through the National Family Planning Coordinating Board (BKKBN) hoped that the method would catch the largest acceptors (2).

Bioconversion of steroid to produce androst-4-en-3,17-dione (AD, see Figure 1) and androsta-1,4-dien-3,17-dione (ADD, see Figure 1) or other steroid compounds from various substrates has been reported repeatedly, especially from sterols (3,4,5,6) and phytosterols (7,8,9). However reports on the solasodine bioconversion are very limited in numbers. Tarigan (10) reported the bioconversion of solasodine alkaloid by various bacteria resulting in the formation of 4,6-solasodien-3-on (solasodienon) and 1,4,6-solasotrien-3-on (solasotrienon). Using several mutants of *Mycobacterium*, Patil and Mishra (11) were able to convert

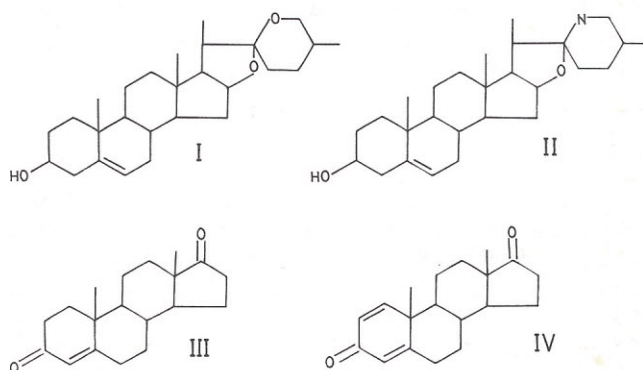


Figure 1. Molecular Structure of diosgenin (I), solasodine (II), AD (III) and ADD (IV).

solasodine into AD and ADD. Of the four mutants tested, *Mycobacterium* sp NRRL B-3805 converted solasodine into ADD with a yield of 10.9%.

Mold, yeast and actinomycetes were also reported by Arima (14) to be able to convert steroid into cholest-4-en-3-on (cholestenon) and cholesta-1,4-dien-3-on (cholestadienon).

The present article reported the results of an explorative study, to establish monitoring methods for the bioconversion of solasodine by *Mycobacterium phlei* DSM 43286 to produce intermediates such as AD and ADD.

MATERIALS AND METHODS

Materials

Mycobacterium phlei DSM 43286 was obtained from Dr Triadi Basuki of the Research and Development Centre for Biotechnology, Indonesian Institute of Sciences, in Bogor. Crude extract of solasodine was kindly supplied by Mr. Kreshna S.A. Head of the R & D Section of PT Kimia Farma, Bandung. Other chemicals and ingredient of culture media were respectively purchased from SIGMA and DIFCO.

Culture starter of *M. phlei*

M. phlei DSM 43286 from slant agar was inoculated into growth medium containing 0.3% beef extract and 0.5% bacto pepton. The mixture was then incubated in an orbital shaker operated at 200 rpm, at 37 °C for 48 hours. The growth curve was observed in order to find the most appropriate time of incubation for further fermentation process. The growth was followed by measuring the absorbancy at 580 nm with 30 minutes interval during the first six hours incubation, and then with a longer time-interval in the further incubation time. Starter for the conversion process was taken from the growth medium after reaching the logarithmic phase.

Bioconversion of Solasodine

The starter (40% volume) was inoculated into the conversion medium containing corn steep liquor (CSL) 0.50%, NH_4Cl 0.30%, KH_2PO_4 0.05%, CaCO_3 0.30%, Na-sitrate 0.30%, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.20%, urea 0.02% and 8-hydroxyquinoline 0.01%. Incubation was carried out at 37 °C in an orbital shaker at 200 rpm.

Analyses

Samples for analyses were taken periodically at a 24 hour interval. Samples were spun to separate the biomass. The filtrate was then extracted thrice with chloroform (9). The volume ratio of the filtrate and chloroform was 1 : 0.5. The chloroform layer was collected and washed with 2% oxalic acid solution to remove 8-hydroxyquinoline and the excess oxalic acid was then washed with water. The chloroform extract obtained was further dried with anhydrous Na_2SO_4 , and then evaporated to dryness.

Identification of the fermentation products was done by thin layer chromatography (TLC) technique. Firstly the optimum separation condition was investigated for standards and samples,

and the condition was then used to identify whether AD or ADD was formed.

RESULTS AND DISCUSSIONS

The growth of *M. phlei* in nutrient broth prepared for starter is shown in Figure 2. The first two hours seems to be the adaptation period which was followed by accelerated growth until hour-3.5. The logarithmic phase was between hour-3.5 and hour-9 and the subsequent stationary phase occurred thereafter. Transfer of

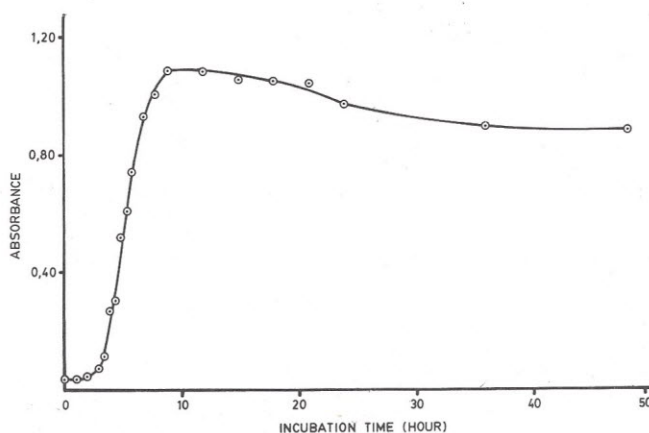


Figure 2. Growth curve of *M. phlei* DSM 43286 in nutrient broth.

bacterial culture into conversion medium was done when the growth reached logarithmic phase (hour-8.5). *M. Phlei* could grow well in conversion medium and the fermentation products were identified by TLC/HPLC and the results were as follows.

The best separation of AD and ADD in TLC silica plates was found when a mixture of chloroform-ethyl acetate (80:20) was used as the eluent. The fermentation broth samples were then analysed using the same separation condition (Table 1). Visualization of spots with iodine vapour gave brown colour with R_f values different from those of AD and ADD standards. Placing the TLC plate under UV lamp at 254 nm did not show any spot. When detection was set at 366 nm, fluorescing spots appeared from samples harvested at day-5, day-6, day-8, day-9, day-10, day-11, and day-12, although AD and ADD themselves did not appear at this wavelength (Table 1).

Maximum absorption of the bioconversion products were found between 231 and 234 nm, which was less than that of AD (239 nm) or ADD (243 nm), but higher than that of solasodine crude extract (205 nm), as shown in Table 2. Molar extinction coefficient (ϵ) of the fermentation samples were less than those of AD, ADD or solasodine crude extract. At 240 nm, which is a wavelength between the maximum one for AD and ADD, ϵ value of the bioconversion samples were in the range of 576 – 4718. This is much lower than ϵ for either AD (20,270) or ADD (21,658), but higher than ϵ for solasodine crude extract (569).

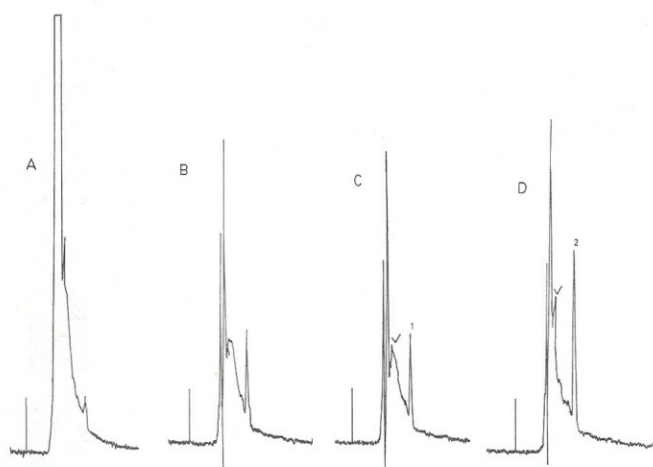


Figure 4. Chromatograms of the solasodine fermentation samples. Column: μ -porasil; Eluent: Benzene-ethylacetate-chloroform (40:80:10); Detector: UV 254 nm.
A: Control C: Fermentation day-2
B: Fermentation day-1 D: Fermentation day-3

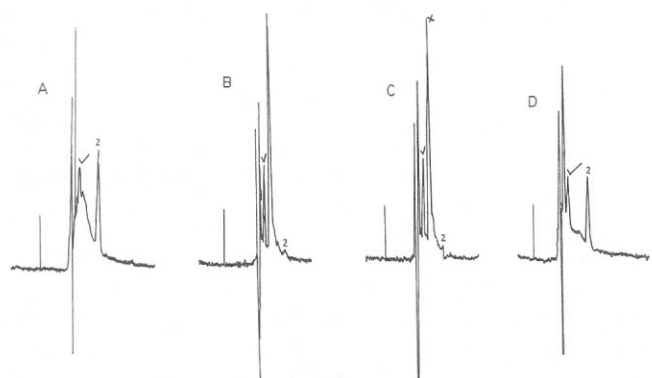


Figure 5. Chromatograms of the solasodine fermentation samples. Column: μ -porasil; Eluent: Benzene-ethylacetate-chloroform (40:80:10); Detector: UV 254 nm.
A: Fermentation day-4 C: Fermentation day-6
B: Fermentation day-5 D: Fermentation day-7

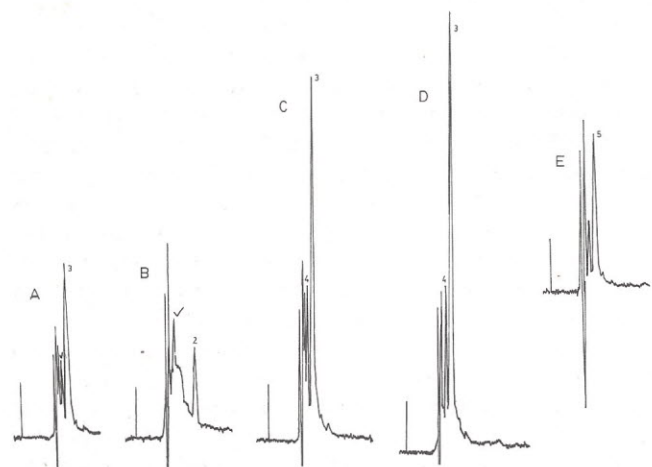


Figure 6. Chromatograms of the solasodine fermentation samples. Column: μ -porasil; Eluent: Benzene-ethylacetate-chloroform (40:80:10); Detector: UV 254 nm.
A: Fermentation day-8
B: Fermentation day-9
C: Fermentation day-10
D: Fermentation day-11
E: Fermentation day-12

Further studies are needed to provide more evidences of the bioconversion process conducted to confirm the formation of AD and ADD from solasodine. Spectrodiodearray detector or LC-MS and other chromatographic and spectroscopic techniques should be examined to obtain sensitive methods for detection of the products formed during the bioconversion of solasodine.

CONCLUSION

The results obtained from the present investigation may suggest that solasodine could be converted perhaps into AD or ADD and other intermediates with the help of the *M. phlei*. However, the optimum condition for the bioconversion needs further investigation. Similarly the most appropriate solvent systems for product separation are still to be worked out. Other analytical techniques such as chromatographic and spectroscopic techniques could be candidates to be examined.

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