ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 7, Issue 4, 2014

ISSN - 0974-2441

Research Article

ENHANCED BIOTRANSFORMATION OF PHYTOSTEROLS, A BYPRODUCT OF SOYBEAN REFINERIES, TO A KEY INTERMEDIATE USED FOR SYNTHESIS OF STEROIDAL DRUGS

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Received: 04 August 14, Revised and Accepted: 09 September 14

ABSTRACT

Objective: During refining of soybean oil, a phytosterols rich fraction referred as deodistillate is the major byproduct. Phytosterols are the source of valuable precursor for the synthesis of steroidal drugs extensively used in allopathic system of medicine. The present work describes the bioconversion of phytosterols to androst-4-ene-3 17-dione, a key intermediate for the production of steroidal drugs and hormones. Since, phytosterols are insoluble in an aqueous medium, efforts are done to increase the bioavailability using some organic solvents as dispersing agent, and the biotransformation was catalyzed by Mycobacterium fortuitum subsp. fortuitum NCIM 5239, a mutant obtained at Devi Ahilya University, Indore.

Methods: Bioconversion reactions were carried out in triplicate shake flask culture. Some water miscible and water immiscible solvents were used as dispersing agents, and a solution of phytosterols in solvents was added prior to autoclaving. The analysis of converted product was done by quantitative thin layer chromatography.

Results: When compared to the bioconversion recorded with micronized phytosterols as a control for substrate addition, increased bioconversion was observed by ethanol as dispersing agent at a concentration of 4 ml per 0.3% substrate.

Conclusion: Dispersing agents dispersed the phytosterol particles, thereby increasing the bioavailability to the bacterial cells in the medium. Ethanol may be used to improve the yield of bioconversion reactions.

Keywords: Androstenedione, Androst-4-ene-3, 17-dione, Mycobacterium fortuitum, Phytosterols, Steroid bioconversion, Soybean refineri.

INTRODUCTION

A majority of steroid drugs as anti-inflammatory, anti-allergic, cardiotonic, geriatric, progestational, anabolic, immunosuppressive and contraceptive agents have been successfully introduced in the allopathic system of medicine, after the realization of powerful anti-inflammatory activity of cortisone in the treatment of rheumatoid arthritis in 1949 [1,2]. The commercial production of these pharmaceutically active steroidal drugs depends upon three C-17-ketosteroid precursors namely, androst-4-ene-3, 17-dione (AD), androsta-1, 4-diene-3, 17-dione (ADD) and 9α hydroxy (9-OH-AD) [3,4]. AD, 17-dione (AD) is a representative member of 17-ketosteroid family; a major starting material for the synthesis of anabolic drugs, testosterone, estradiol, ethinylestradiol, testolactone, progesterone, cortisone, cortisol, prednisone, prednisolone and oral contraceptives [5].

The microbial cleavage of C-17 side chain of cholesterol and various phytosterols (Fig. 1) have been reported [6-8]. These degradation of C-17 side chain of phytosterols yields AD and ADD; two key intermediates used for commercial production of the majority of medically important steroids. Phytosterols are the abundant source for transformation into these precursors as they are byproducts of many vegetable oil refineries and wood pulp industries [9,10]. Deodistilate, a phytosterols rich fraction is a major byproduct of soybean oil refineries. From deodistilate, phytosterols are separated and used as raw material in pharmaceutical industries. However, steroids and sterol compounds are sparingly soluble in water, usually below 0.1 mM and 1 μM respectively; that low solubility of steroid substrates imposes a barrier for the bioconversion reactions by microorganisms [11]. Various research groups have tried to increase bioavailability of sterol substrate by reducing particle size by substrate micronization [12], by using organic solvents like dimethylformamide [13], dimethylsulfoxide, hexane, toluene etc. [14-16]. Some of them, used vegetable oil as substrate carrier [17], tweens [18,19], triton X 100, lecithin [20,21],

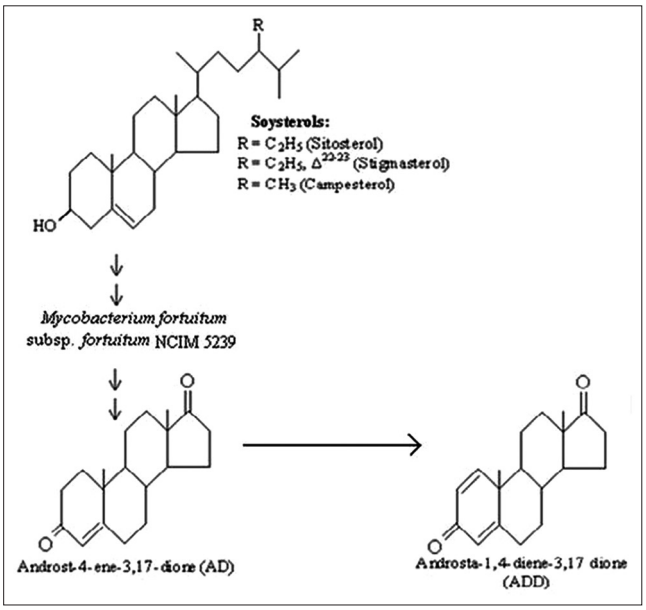

Fig. 1: Schematic illustration of biotransformation of phytosterols to androst-4-ene-3, 17-dione and androsta-1, 4-diene-3, 17-dione key intermediates required for majority of steroidal drugs

to enhance the bioconversion by microorganisms. Mycobacterium fortuitum subsp. fortuitum NCIM 5239 possesses ability to cleave C-17 side chain of soysterols to yield AD [22]. The present work was carried out to study the effect of some water miscible and water immiscible organic solvents on the bioconversion of phytosterols to AD by M. fortuitum subsp. fortuitum NCIM 5239.

METHODS

Strain

The Strain used in this study was M. fortuitum subsp. fortuitum NCIM 5239, a mutant of M. fortuitum subsp. fortuitum MTCC 929 [22]. The strain was maintained on nutrient agar at 4°C.

Materials

Chemicals and solvents were purchased from different suppliers. AD, 17-dione (AD) was purchased from sigma chemicals, USA. Acetone, methanol, 1,2-propanol, chloroform and ethyl acetate were procured from Merck, India. Ethanol (Bengal Chemical, India) and 1-butanol (Glaxo Lab, India). All solvents used in the study were of LR grade. Phytosterols was gifted by Sonic Biochem Extractions Pvt. Ltd., Indore, India.

Addition of phytosterols

Substrate was dissolved in the organic solvent by heating on a boiling water bath, transferred on a magnetic stirrer and 25 ml warm incubation medium was slowly added with continuous stirring. Alternatively micronized suspension of phytosterols was prepared by adding phytosterols in incubation medium along with 30 gm glass beads in Erlenmeyer flask and shaking it on gyratory incubator shaker. After 30 minutes of incubation, 25 ml medium was dispensed in three flasks of 150 ml capacity and sterilized in an autoclave.

Bioconversion and extraction of product(s)

The inoculum was prepared in 250 ml optimized seed medium B [22] containing g/l: Glycerol, 12.68; urea, 1.06; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.5; $FeCl_3.6H_2O$, 0.05; meso-inositol, 0.0667; pH adjusted to 7.0 in 500 ml capacity flask. After sterilization at 121°C for 15 minutes in an autoclave, the medium was inoculated with 1 ml actively growing M. fortuitum subsp. fortuitum NCIM 5239 and incubated on a gyratory incubator shaker (300 rpm, 32±2°C) for 48 hrs. Bioconversion of phytosterols was initiated by addition of 5 ml inoculum in 25 ml sterile medium of same composition in 150 ml capacity flask described above. The flasks were incubated on a gyratory incubator shaker (300 rpm, 32±2°C) and the samples of broth were aseptically drawn after regular intervals of 48 hrs. 1 ml broth was extracted with 1 ml chloroform and organic phase was analyzed for product(s) formation.

Analytical method

The bioconversion of phytosterols to AD was observed by quantitative thin layer chromatography (TLC) as described by Gulla et al. (2008) [23] on pre-coated silica gel plates (Merck-1.05554.007, Darmstadt, Germany). 1 ml fermentation broth was extracted with 1 ml chloroform, the separated organic phase after centrifugation was dried over sodium sulfate and 10 µl of the chloroform extract of bioconversion medium was spotted along with three concentrations (0.5, 1 and 5 µg) of authentic AD prepared in chloroform. The TLC plates were developed in ethyl acetate: benzene (4:5) and spots were visualized by spraying the plates with 2% ceric ammonium sulfate in 60% sulfuric acid, followed by heating at 110°C for 5 minutes. The plates were scanned on Samsung SCX-4100 scanner and AD was quantified by Image quant TL Software (G.E. Healthcare Life Sciences, India) version 7.0 (Fig. 2).

RESULTS

Water miscible organic solvents as dispersing agents

Water miscible solvents like acetone, methanol, ethanol and 1, 2-propanol have been used for the process. Mole percent conversion of phytosterols to AD increased up to 144 hrs and then decreased further. As compared with control; acetone and propanol showed minor accumulation of conversion product, whereas other solvents ethanol and methanol has shown 22.62%, 19.18% mol percent conversion respectively. Fig. 2 shows mol % bioconversion of phytosterols to AD using water miscible and water immiscible organic solvents as dispersing agents. Control showed 18.84% mol conversion at 144 hrs (Fig. 3).

Water immiscible organic solvents as dispersing agents

Water immiscible solvents including butanol, chloroform and ethyl acetate have been used for the process. Butanol and chloroform exerted an inhibitory effect on the bioconversion during the initial phase of bioconversion up to 96 hrs. Compared to other solvents, ethyl acetate showed higher conversion (14.53 mol %) at 192 hrs. Control exhibited higher accumulation of bioconversion product at 144 hrs (Fig. 4).

Optimum concentration of ethanol

As ethanol showed better conversion than the micronized substrate, optimum concentration of ethanol has been determined. After 144 hrs incubation period, significant increase in AD content of the medium was recorded over 1 ml ethanol (E1) using E3, E4, E5 which were 3 ml, 4 ml and 5 ml ethanol, respectively. Accumulation of AD increased with the concentration of solvent up to a certain extent. E4 had been showed enhanced bioconversion with 53.35% mole

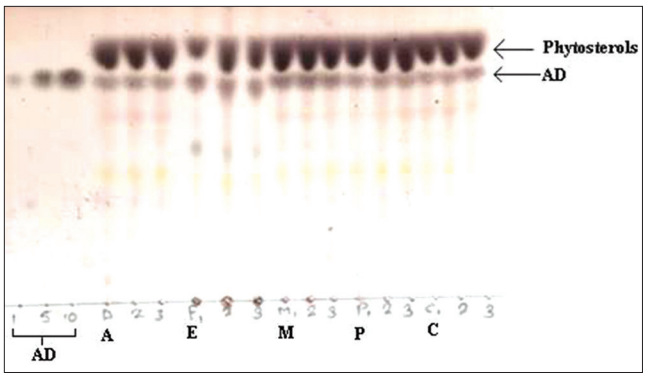

Fig. 2: Scanned image of thin layer chromatography plate showing bioconversion of phytosterols to androst-4-ene-3, 17-dione (AD) (greenishblue spots), substrate dissolved in acetone (A), ethanol (E), methanol (M), propanol (P) and control (C) along with authentic sample of AD (1,5, and 10 µg)

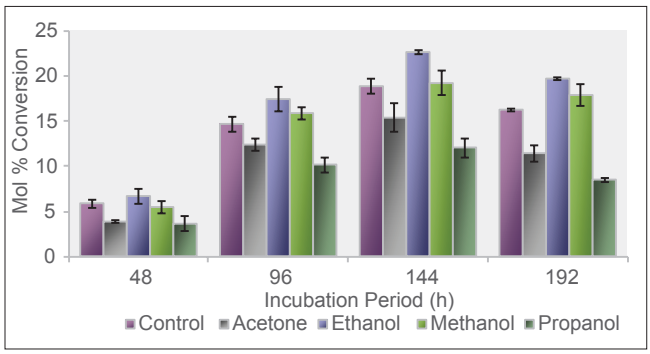

Fig. 3: Mol % conversion of phytosterols to androst-4-ene-3, 17-dione using water miscible organic solvents as dispersing agents

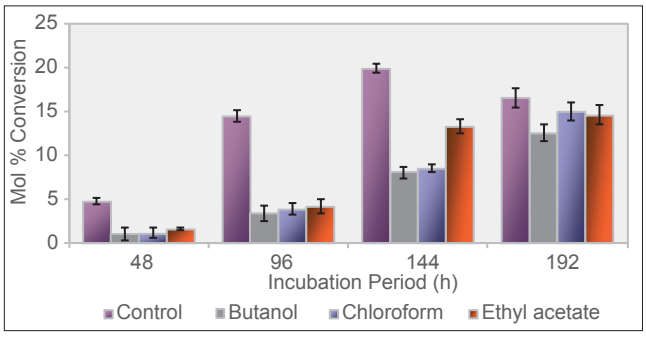

Fig. 4: Mol % conversion of phytosterols to androst-4-ene-3, 17-dione using some water immiscible organic solvents as dispersing agents

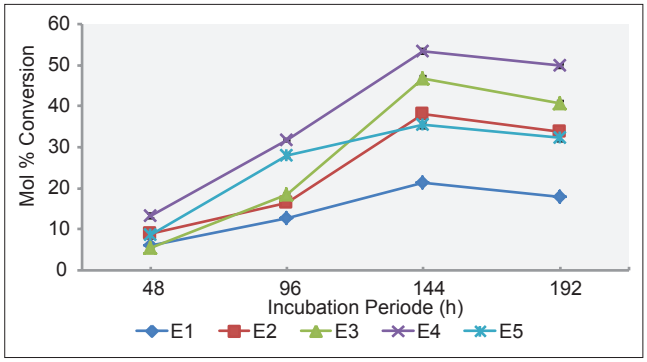

Fig. 5: Mol % conversion of phytosterols to androst-4-ene-3, 17-dione using different concentration of ethanol (1 ml-E1, 2 ml-E2, 3 ml-E3, 4 ml-E4, 5ml-E5; with standard deviation)

conversion. Higher concentration of ethanol inhibited the present bioconversion (Fig. 5).

DISCUSSION

Hydrophobic nature of sterol particles makes them unable interacts with organisms growing in an aqueous medium. Even micronized substrate tend to clump and disperse poorly, resulting in the reduction of product yield [24]. The methodology needed is to disperse the phytosterols in medium forming fine particles of sterols, which are easily adsorbed on the lipid bilayer of microorganisms. Different methodologies were adopted like use of organic-aqueous, different emulsifiers, detergents and two-liquid phase systems which disperse phytosterols.

Unlike other solvents, dispersing agents added prior to autoclaving. That causes evaporation of some amount of solvents from medium leaving dispersed substrate behind. Though organic solvents disperse the phytosterols in medium, they exhibit toxicity on microorganisms. They solubilize the cell wall of bacteria as well as subsequently lysis of the cell [25]. The present work indicated that ethanol exhibits low toxicity at lower concentration. Except ethanol, all other organic solvents exhibits toxic effect on the cell. Solvents also effect catalytic activity and the enzyme activity causing decrease in bioconversion ability of organisms [26,27].

CONCLUSION

Steroid bioconversion reactions are limited by the fact that the substrate is highly hydrophobic and the bioconversion reaction is catalyzed in a hydrophilic environment. Hence, there is a need of technology that improves the degree of biotransformation that helps to reduce the final cost of steroidal drugs. The present work illustrated that the ethanol at optimum concentration may be used to disperse hydrophobic sterol substrates to enhance the degree of bioconversion. The results exhibited that a multiple analysis is required when it is envisaged to correlate the effects of solvents in biotransformation of phytosterols.

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