Australian Journal of Basic and Applied Sciences, 4(9): 4107-4112, 2010
ISSN 1991-8178

Enhancement of β-sitosterol Bioconversion by *Fusarium solani* Using Aqueous-organic Solvent System

El Refai,H.A. and Abd-elslam, I.S.

Natural and Microbial Products Chemistry Dept., National Research Centre, Dokki, Cairo, Egypt.

Abstract: The selective cleavage of the β--sitosterol side-chain by the free cells of *Fusarium solani* was studied to detect the combined effect of both solvent nature and concentration in two-phase aqueous organic medium. This multi-step degradation pathway of sterols leads to the production of 4-androstene-4, 17-dione (AD) and 1, 4- androstadiene 3, 17-dione (ADD). The bioconversion was strongly affected by the nature and by the aqueous/ organic phase ratio. Some androstenes are the key intermediates of microbial side-chain cleavage of phytosterol which is an alternative to the multistep chemical synthesis. An attempt was made in this study to improve the substrate-biocatalyst interaction in order to increase the bioconversion yield. Comparison study was held between n-hexane and toluene to study their effects on the bioconversion process in batch fermentation technique. The results showed that maximum AD and ADD yields (53.3,25% and 58.7,25.9%) was obtained by the addition of 10% n- hexane or toluene respectively to the production medium, at substrate concentration 30 mg/100ml medium, supplemented with 2 ml tween 80 and 0.2mg % glycine.

Key words: bioconversion, sterols, organic solvent, *Fusarium solani*

INTRODUCTION

Microbial conversion of natural sterols (cholesterol or phytosterols) to steroidal precursors has long been used for the synthesis of steroidal drugs of biomedical importance (Kieslich, 1985;Mahato and Garai, 1997). These include the production of several higher-value steroidal compounds derived from 1,4-androstenedione (AD) and 1,4 androstadiene 3,17 dione (ADD), such as progestational, adrenocortical, estrogenic and contraceptive agents. Current research focuses on the development of methods that improve hydrophobic substrate biocatalyst interaction to increase bioconversion yield.

The concept of aqueous organic two liquid phase bioreactive systems with whole microbial cells has been found to effectively enhance the yield of several bioprocesses of commercial interest (Panke *et al.,* 2002; Stark and Stockar, 2003). The organic phase enables the storage of high concentration of substrates and / or product. The most implementation factor of an effective aqueous-organic bioconversion in the selection of a proper solvent, which has to be biocompatible and to provide an adequate substrate reservoir and product sink (Leon *et al.,* 1998; Macleod and Daugulis, 2003).

This paper is an effort to enhance the bioconversion yields of *β*-sitosterol using the local isolate *Fusarium solani* in aqueous-organic solvent phase to enhance the water insoluble substrate utilization for the improvement of *β*-sitosterol side chain degradation reaction.

MATERIALS AND METHODS

Microorganism:

Fusarium solani NRC 105 was obtained from the culture collection of the Natural and Microbial Products Chemistry Department, National Research Centre (NRC), Dokki, Cairo, Egypt.

Chemicals:

The authentic steroids used in the current work (AD, ADD and β-sitosterol), and 8-hydroxyquinoline were provided by Sigma Company USA. The other chemicals were obtained from (Merck) , all the solvents used are HPLC grade.

Corresponding Author: El Refai,H. A, Natural and Microbial products Chemistry Dept., National Research Centre, Dokki, Cairo, Egypt.
Abdelsalam66 @hotmail.com

***Maintenance of the microorganism*:**

The experimental organism was maintained on the following medium (g/l) malt extract 25, yeast extract 4 and agar 20 and was monthly interval recultured.

Transformation Process:

Cultivation was performed using 250 ml Erlenmeyer flasks, each containing sterile 100 ml of the following medium (g/l): glucose 10, $(NH_4)_2$ SO_4, 1, K_2 HPO_4 ,3 and 8- hydroxyquinoline 0.016 to avoid the enzymatic cession of the sterol nucleus (Nagasawa *et al.,* 1970). Each flask was inoculated by 4ml of 48 hr old culture, growth continued on a reciprocal shaker 200 rpm at 30°±1 for 72 h, pH 6.5±, thereafter to each flask 10 ml of a given solvent was added according to the method described by (El-Refai, 2002). The substrate (β-sitosterol) was added to each flask at a given concentration, and the transformation process was continued for the required time.

Extraction:

At the end of the transformation period, the content of each flask was extracted with double its volume of chloroform. The solvent layer was separated and dried under vacuum to give semi solid residue "test material".

Qualitative Analysis:

The test material was dissolved in a measured volume of chloroform: methanol (1:1 v/v). Analysis was carried out by thin layer chromatography (TLC), using silica gel G_{60} and elution solvent n-hexane: diethylether: glacial acetic acid (70:30:1 v/v/v). The transformation products were identified by studying the TLC profile of each compound with that of the authentic samples using Libermann Burchared reagent as colour reagent (Sallam *et al.,* 2007).

Quantitative Analysis:

The gas liquid chromatography technique (HPLC) technique was adopted for preparing the steroid derivatives. Aliquots of 0.1ml of the tested material was dried under nitrogen stream. The trimethyl-o-methyloxime derivatives were synthesized by the method described by (El-Refai, 2002). The bioconversion percentage was calculated as follows:

$$\text{AD or ADD \%} = \frac{\text{amount of AD or ADD detected}}{\text{amount of β-sitosterol added}} \times 100$$

RESULTS AND DISCUSSION

Comparison of different organic solvents effect on microbial side chain cleavage of *β*-sitosterol to AD and ADD by *F. solani* was tested in an aqueous / organic solvent system (10:1 V/V) as shown inTable (1). Maximum yields (38.2, 40.5%) for AD and (18.5, 17.9%) for ADD were obtained using n-hexane and toluene as organic solvent respectively at transformation period extended to 72hr.

These two solvents possessed the highest log p value which is defined as the logarithm of the partition coefficient of the solvent in octanol-water-two-phase system. Other solvents showed lower AD and ADD yield, these solvents have lower log p values. The correlation between the activity of microorganism and log p was stated by (Laane *et al.,* 1987), where solvents having log p value below 2 are relatively polar and not suitable for biocatalytic activity. On the other hand, solvents having log p values above 4 exhibited satisfactory biocatalytic activity. The level of biocatalytic activity is intermediary in solvents with log p values between 2 and 4.

Substrate Tolerance Studies:

Tolerance of the fungus cells to high β,sitosterol concentrations has been studied using β-sitosterol concentration from (10-50 mg %) results shown inTable (2) predicted the possibility of increasing concentration of poorly water soluble substrate (β, sitosterol) using aqueous/ organic phase system. The production of AD and ADD in an aqueous toluene two phase system was higher than that in an aqueous hexane two phase system. The reason may be due to the better partition coefficient of AD and ADD in toluene phase than in n-hexane phase.

The best AD and ADD yield (43.3 and 20.4 %) respectively were obtained in aqueous toluene two phase using 20 mg% β-sitosterol, good stability of side chain degradation system of *F. solani* was shown on using higher concentration of the sterol as substrate up to 50 mg/% and the fungus could undergo the

biotransformation process. Similar results were predicted by (Leon *et al.,* 1998; Macleod and Daugulis, 2003; Nagasawa *et al.,* 1970; El-Refai *et al.,* 2002; Sallam *et al.,* 2007; Laane *et al.,* 1987 and Cruz *et al.,* 2003) who reported that the water immiscible organic phase acts as reservoir for both substrate and product.

The biotransformation product is transported to the organic phase because of its significantly higher solubility in that phase, and consequently, recovery of product is easy.

Table 1: Effect of different solvents on the bioconversion of β-sitosterol by *F.solani*

Solvent	Log *p* value	Transformation products %	
		AD	ADD
* Control	-	30.5	10.7
Chloroform	0.2	35.7	12.2
n-hexane	3.5	38.2	18.5
Toluene	2.5	40.5	17.9
Ethylacetate	0.68	25.3	11.5
Petroleum ether	ND	18.1	15.3
n-Butanol	0.8	15.5	-
Ethyl alcohol	2.1	30.4	11.2

*Control : no solvent used
* solvent conc. (1:10 v/v) organic:aqueous
* Substrate conc. 5mg/ 100ml media
* Transformation time 72 hr.

Table 2: Effect of different substrate concentration on the bioconversion of β-sitosterol by *F.solani*

Substrate conc.mg/100ml	n.hexane transformation Products%		Toluene transformation Products%	
	AD	ADD	AD	ADD
10	38.2	18.5	40.5	17.9
20	41.5	20.7	43.3	20.4
30	30.5	11.6	31.4	12.5
40	21.2	8.5	23.9	10.3
50	15.5	7.3	18.3	9.5

Effect of Different Transformation Period on the β-sitosterol Biotransformation:

The transformation process was carried out for different time intervals (24, 48, 72, 96 hr) at which the fermentation was terminated and the products were analyzed. AD and ADD production were evidently affected by the time at which the fermentation extended (Fig.1). Maximum yields were obtained after 48 hr. The production of AD and ADD (43.7, 22.3%) were obtained by using n-hexane. However, (45.7% and 24.1%) of the products were achieved by using toluene respectively. As transformation period extended a remarkable decrease in the products outputs were observed. These results were also reported by (Noh and Kim, 2000; Perezm *et al.,* 2006 and Koryeka *et al.,* 2001).

Attempts to Overcome the Substrate Low Solubility Using Some Additives for β, Sitosterol Bioconversion: A-addition of Gylcine:

Glycine which is known to create controlled defects in the fungus cell wall (Koryaka-Machala *et al.,* 2005; Malaviya and Gomes, 2007), these defects in the cell wall appear to enhance *β*-sitosterol side-chain cleavage (Sediazak *et al.,* 1999). Glycine affects also the peptidoglycan layer by reducing cross-linking levels between peptide layers. In this experiment different concentrations of glycine were tested (0.1, 0.2, 0.3 g/l). Comparing the results (Fig.2), revealed that the conversion of the sterol to AD and ADD (53.3 and 25%) using n-hexane as organic solvent was enhanced by using (0.2 g/l) glycine. On the other hand, toluene affects the bioconversion process in higher manner, where the best yields of AD and ADD were (58.7 and 25.9%) respectively.

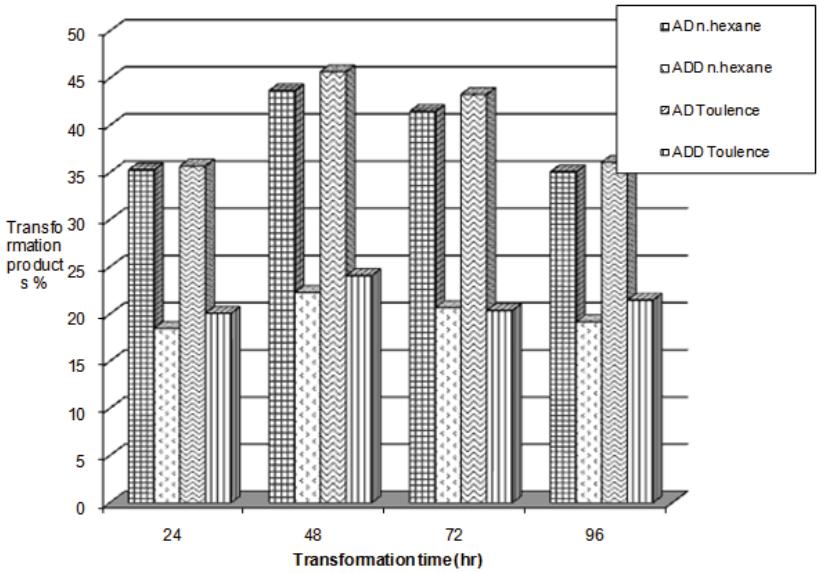

Fig. 1: Effect of different transformation time on the bioconversion of β,sitosterol in aqueous organic two phase system

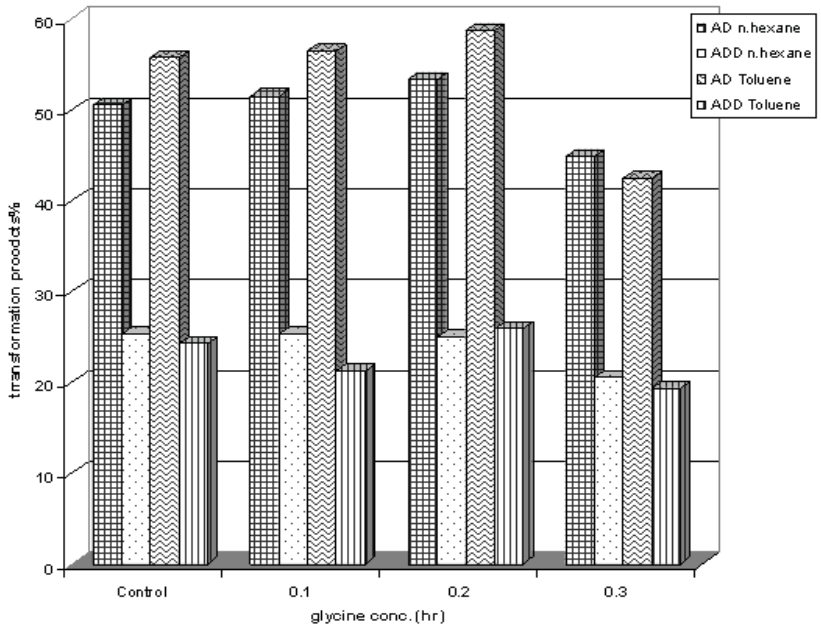

Fig. 2: Effect of different glycine concentrations on the bioconversion of β,sitosterol in aqueous organic two phase system

b. Addition of tween 80:

The effect of tween 80 has been assessed (Korycka *et al.,* 2005). This agent increased the transport across cell barrier by enhancing the substrate solubilization in media surrounding the cell, consequently the concentration gradient. Surfactants also increased the permeability of the membrane and enhances the specific activity of sterol side-chain cleavage to AD and ADD (Rumijowaska *et al.,* 1997; Cruz *et al.,* 2002). The present investigation involved using different tween 80 concentration were (1,2,3,4%). The results (Fig.3) showed that the best sitosterol conversion was attained using toluene solvent were the transformation products yield were (60.7 and 24.3) for both AD and ADD respectively.

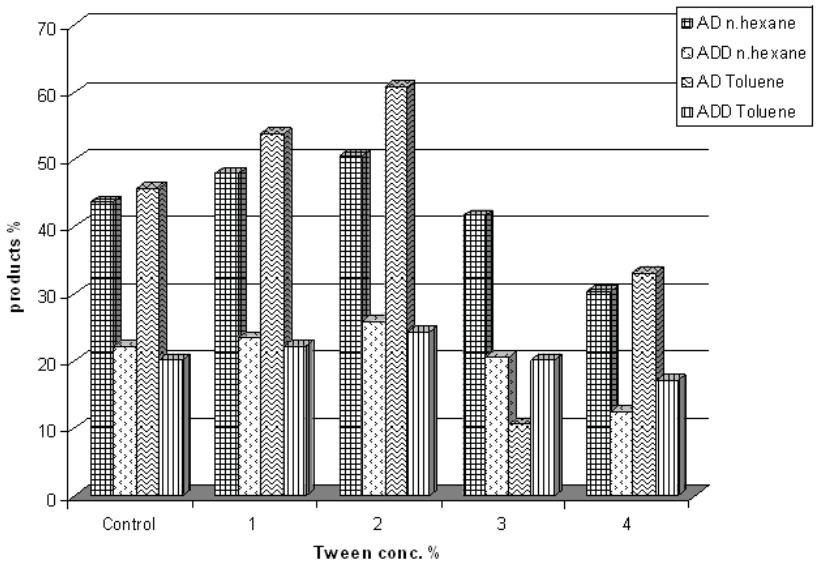

Fig. 3: Effect of different tween 80 concentrations on the bioconversion of β,sitosterol in aqueous organic two phase system

REFERENCES

Kieslich, K., 1985. Microbial side chain degradation of sterols. J Basic Microbiol., 25: 461-469.

Mahato, S.B., S. Garai, 1997. Advances in microbial steroid transformation. Steroids., 62: 332-345.

Panke, S., M. Held, M.G. Wubbolts, B. Wiltholt, A. Schmid, 2002. Pilot-scale production of (S) styrene oxide from styrene by recombinant Escherichia coli synthesizing styrene monooxygenase. Biotechnol Bioeng; 80: 33-4.

Stark, D., U. van Stockar, 2003. In situ production removal (ISPR) in whole cell biotechnology during the last twenty years. Adv Biochem Eng/Biotechnol, 2000. 80: 150-75

Leon, R., P. Fernandes, H.A. Pinheiro, J.M.S. Cabral, 1998. Whole-cell biocatalysis in organic media. Enzyme Microb. Technol., 23: 483-500.

MacLeod, C.T., A.J. Daugulis, (2003. Biodegradation of polycyclic aromatic hydrocarbons in a two-phase partitioning bioreactor in the presence of a bioavailabe solvent. Appl. Microbiol. Biotechnol. (published on line 12 April 2003).

Nagasawa, M., H. Hasniba, N. Watanabe, M. Bae, G.Smura and K. Arima, 1970. Microbial transformation of sterols part III cleavage of sterols side chain by microorganisms.

El-Refai, H. and I.S. Abd-Elsalam, 2002. Sucessful conversion of β, sitosterol and crude sterol by local isolated *Fusarium solani* .Al Azhar J. Microbial., 55: 114-122.

Sallam, L.A., M. Osman, A. Hamady, G. Zaghlol, 2007. Microbial transformation of phytosterals mixture from rice bran oil unsaponifeable matter by selected bacteria**.** World J. Micobiol. Biotechnol., (10): 9689-95.

Laane, C., S. Boeren, K. Vos, C. Veeger, 1987. Rules for optimization of biocatalysts in organic solvents. Biotechnol Bioeng., 30: 81-7.

Cruz, P., J.M.S. Fernandes, H.M. Cabral, Pinheiro, 2003. Solvent partitioning and whole-cell sitosterol bioconversion activity in aqueous-organic two-phase systems. Enzyme Microb. Technol., 34: 342-353.

Caroline, S., T. Factebour and J. Bensson, 1999. Metabolism of β sitosterols by *pseudomonas sp*. Photochemistry, 52: 1279-1282.

Noh, S. and K. Kim, 2000. Conversion of sterols into AD and ADD by *Mycobacterium fortuitum* mutant. (Eugene-science Inc., S. Korea) PCT Int. App. Wo0292,830: Cl2p331.

Perez, C., A. Falero, H.L. Duc, Y. Balcinde, B.R. Hung, 2006. Bioconversion of phytosterols to androstanes by *mycobacteria* growing on sugar cane mud. J. Ind. Microbiol. Biotechnol, 148: 719-723.

Korycka-Machala, M., A. Ziolkowski, A. Rumijowaska-galewicz, K. Lisowaska, L. Sedlaczek, 2001. Polycations increase the permability of *Mycobacterium vaccae* cell envelopes to hydrophobic compounds. Microbiology, 147(2769): 2781.

Korycka-Machala, M., A. Galewiez-Rumijowska-Galewicz, J. Dziadek, 2005. The effect of ethambutol on *mycobacterial* cell wall permeability to hydrophobic compounds. Pol. J. Microbiol., 54: 5-11.

Malaviya, A., J. Gomes, 2007a. cell wall permeabilizing agents as the potent enhancer of biotransformation of sitosterol to androstenedione. In: Proceedings of Biomicro World 2007, Sevilla, Spain, 385.

Sedlazek, L., K. Lisso waska and Dhegonska, 1999. The effect of cell wall component on glycine enhanced sterol side chain degradation to androstene derivatives by *Mycobactorium.* Appl. Microbiol. Biotechnol. 52: 563-571.

Rumijowaska, A., K. Koswaka, A. Ziokowski, L. Sedlaczek, 1997. Transformation of sterols by *Mycobacterium vaccae*: effects of lecithin on the permeability of cell envelopes to sterols. World J. Microbiol. Biotechnol., 13: 89-95.

Cruz, A., P. Fernandes, M.J.S. Cabral, H.M. Pinherio, 2002. Effect of phase compositon on the whole-cell bioconversion of β-sitosterol in biphasic media. J Mol Catalysis B., 19/20:371-5.