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Utilization of some sterols derived from *Jatropha crusa* as a substrate for production of androstiendion (AD) and androstiendindione (ADD): maximization using isolated strain of *Absidia corymbifera*

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ABSTRACTS

The growing demands on the steroids encouraged searching for new sources. Jatropha sp. is rich sterols. The common sterols included β-sitosterols, campsterol and stigmasterol. An isolated strain of Absidia corymbifera showeda potentiality to transform some sterols to important steroids, Androsteindione (AD) and Androstadienedione (ADD) as a major products. These are medically important in steroids. An optimization depends on two levels factorial design as a screening forthe effective factors from seven cultural factors and three levels model for optimization of the effective factors were used. The most significant factors found to be the molasses concentration, time and temperature. These factors were further optimized using the three levels factorial design depending on polynomial quadratic equation. The products yields were 32 % for ADD and 15 % for AD at the beginning of the optimization process. After the statistical optimization the yields reached 77 % for ADD and about 18% for AD.

Keywords: *Abisidia corymbifera*, Androsteindione, Androstadienedione.

INTRODUCTION

Sterols are the natural, organic compounds that arewidely distributed in all eukaryotic organisms. The sterols are a part of the cell membranes composition[1]. Important compounds like steroid hormones and bile acids in humans, brassinosteroids - phytohormones in plants are derived from sterols [2]. The sterols are involved in important growth and developmental processes in most living organisms [3].The most common phytosterols are sitosterol, campesterol and stigmasterol. There are other phytosterols like Δ^5avenasterol and cycloartenol are relatively present in a smaller amounts [4].

Androsteindione (AD) is a compound has structural and pharmacological resemblance to testosterone hormone. Its cane be considered an anabolic steroid. It is common for the anabolic steroids to be used to treat hormone deficiency and muscular atrophy caused by the onset of cancer or HIV [5]. It is able to increase the levelof blood testosterone, the body strength, the lean mass and improve the sexual performance. AD possesses many essential properties of an androgen such as binding to the ligandbinding domain of the androgen receptor, induction of its nuclear translocation, and promotion of myogenic differentiation. [6,7].One of the most important use is to act as a starting material for preparation of androgens and anabolic drugs and recently for the production of spironolactone. Other compoundschemically and pharmacologically related to AD.Androstadienedione (ADD)isused as a precursor for preparing pharmaceutically-interesting steroids such as estradiol or estrone[8, 9]. It is commerciallyproduced by the microbiological transformation ofβ-sitosterol and cholesterol [10]. The microbial cleavage of C-17 side chain of cholesterol and variousphytosterolshave been reported [11,12, 13,]. These degradationof C-17 side chain ofphytosterols yields AD and ADD; two keyintermediates used for commercial production of the majority

ofmedically important steroids. Phytosterols are the abundant source fortransformation into these precursors as they are byproducts of manyvegetable oil refineries and wood pulp industries [14].

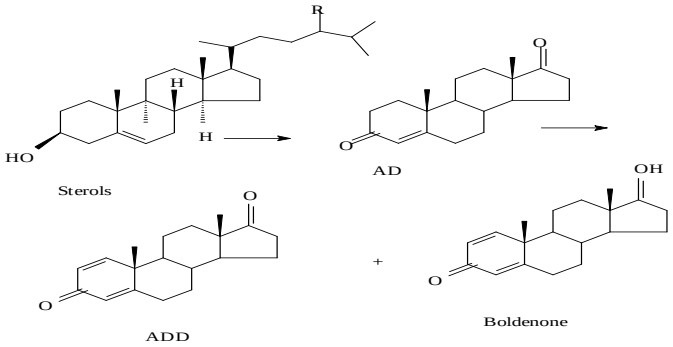


Fig (1). Conversion of sterols to important steroids

Production of ADD) by biotransformation of sterols has not been reviewed exclusively. However, there is extensive literature on biotransformation of sterols to steroidal compounds [15, 16, 17, 18, 19,20].

MATERIALS AND METHODS

1. Extraction and analysis of the sterols.

The total lipid content of the whole plant parts, leaves, stems, fruits, seeds, wasextracted according to the method of Folch, et al [21]. The total plat parts were ground to fine powder. The powder (100gm) was mixed with chloroform/ methanol 2:1 to 20 time volume. The whole mixture was homogenized for 2 h on shaking water bath at room temperature. The homogenate was then filtered with funnel and filter paper (Whatman no.1). The solvent was washed with distilled water and centrifuged at 4000rpm for 10min. the organic phase was separated. The solvent phase was concentrated using Rota vapor under vacuum. The result wasexpressed as the percentage of lipids in the composition. Unsaponifiable matter was determined.About 10g of *J. cursa*lipid was placed into a roundbottomed flask and 30mL ethanol and 5mL of aqueous KOH solution were added with some boiling stones into the roundbottomed flask. After refluxing for 1 hour. The heating was stopped and the reaction mixture wastransferred into the separating funnel. The flask was washed with 10mL ethanol followed by 20mL warm distilled water and then 20mL cold distilled water. The contents of the separating funnel were left to cool at room temperature, after that 50mL of hexane was added into the separating funnel. After shaking the mixture vigorously for 1min, the mixture was left a few minutes to get two phases. The soap solution phase was converted completely into thesecond separation funnel, and 50mL of hexane was added into the separating funnel. After shaking the mixture vigorously for 1 minute, the mixture was left a few minutes to get two phases. The extractions using 50mL of hexane were repeated five times. The combined extracts in the separating funnel were washedthree times with 25mL of 10% (v/v) ethanol, after vigorous mixing in the separating funnel; the ethanol layer was drawn off after each wash.The hexane was evaporated to dryness under the vacuum using a rotary evaporator, the drying was completed in a vacuum oven at 75-80°C, and was cooled in a desiccator and was weighed. This residue is considered the substrate for the biotransformation process. The residuewas dissolved in 50mL 95% ethanol and finally analyzed for the sterols contents.GC analysis was carried out using a Varian 3300 gas chromatography equipped with flame ionization detection system and a non polar capillary column of 60 m × 0.32 mm

, film thickness 0.25 µm. The operating conditions were as follow: The temperature was from 260 to 300 ºC at 4 ºC / min then kept at 300ºC. Nitrogen gas was used as a carrier with flow rate (1ml/min). The substrate was injected dissolved in CH_2CL_2.

The percent of the common phytosterols, campsterol, β-sitosterol, stigmasterol and Δ^5-avansterol was calculated (Table 1).

2- Isolation of the fungal strains.

A soil sample mixed with debris of vegetation (10g) from rural area south of Egypt, was suspended in 90 ml sterile distilled water. The suspension was placed on rotary shaker at 25c for 24 h. thereafter 1 ml of the suspension was placed on plat containing medium composed of (g%): $(NH_4)_2SO_4$, 0.2; KCl, 0.3; NaCl, 0.1; $MgSO_4$, 0.2; KH_2PO_4, 0.02 and 0.1 substrate and chloramphenicol to prevent the bacterial growth. After incubation for 3 days at 25ºC the growing colonies were purified and transferred to plates of Potato dextrose agar media. The isolates were kept refrigerated. The purified colonies were identified according to the morphological characteristics. The most potent fungal strain was further identified by molecular techniques.

3- Screening and the biotransformation process.
Fractions of 50 ml of PDA in 250ml conical flasks were inoculated with 2ml of cell suspension ($4x10^4$ cfu) determined by plat count technique. The flasks were then incubated at 25º C for 48h, then the substrate (0.1%) dissolved in 1ml ethanol was added and the incubation conducted to another 24h.

4- Analysis of the biotransformation product.
After completion of the biotransformation time the contents of each flask was extracted with double of its volume of chloroform and checked. The organic phase was separated using separating funnel and was dried over sodium sulfate anhydrous. The solvent phase was evaporated under vacuum to dryness. The residues were considered the test material. The test material were analyzed for the biotransformation products.The biotransformation products were separated by TLC with comparison with authentic under UVlight and after visualization with sprayingwith H_2SO_4 and heating for 10 min where AD took green color and ADD took red colorBiotransformation products were isolated by HPLC, HP, Agilent series 1100 (USA). Shimadzu, PL Hi-Plex pb column). Samples were analyzed under following condition: the flow rate was set at 4.0 ml/min at room temperature, mobile phase was composed of methanol–water in the ratio 90:10 (v:v). The wavelength was set at238 nm.

5-Identification and characterization of the fungal strains.
The fungal strains were identified according to the morphological characteristics. The identifications relied on colonial and microscopic characteristics[22, 23, 24, 25].

6- The medium.
Seven different media were examined for production of the desired biotransformation products using the selected fungal species. These medium were given the following codes, MI, MII, MIII, MIV, MV, MVI and MVII. The media have the following composition (g%): MI (Potato dextrose agar): potato, 2, Dextrose, 2; MII (Dox's medium): Sucrose, 3, $NaNO_3$, 0.2, K_2HPO_4, 0.1, KCl, 0.05, $MgSO_4$, 0.05 and $FeSO_4$, traces; MIII (Yeast extract medium): yeast extract, 3 and peptone, 5; MIV: Molasses, 10, Yeast extract, 0.3, MnSO4, 0.004, Sod. citrate, 0.001 and K_2HPO_4, 0.4, MV:(Sabroud's dextrose agar): $(NH4)_2SO_4$, 0.2 KCl, 0.3, NaCl, 0.1, $MgSO_4$, 0.2, KH_2PO_4,0.02 and glucose, 2, MVI (Malt extract medium): Malt extract, 3 and peptone 5, MVII (Corn meal media): Corn infusion, 5.

7- Maximization.
The maximization was carried out through two steps, the first one is screening for seven different factors including the culture conditions. The factors screening determined the most significant factors and it was performed using two levels(+) and (-).To evaluate seven different cultural parameters 16 runs were applied the number of runs = 2^{n-3} where n is the number of variables. In addition, the second step was concerned with prediction of the maximum yields that can be achieved. Box-behnken model was used to optimize the conditions for the most significant factors [26]. Each factor was evaluated at three levels (+), (0), (-). The relation between the variables was expressed by second order polynomial equation for the data obtained from the runs design. The equation is as follow:

$$Y = b0 + b1X1 + b2X2 + b3X3 + b11X\,1 + 2b22X2 + 2b33X3 + 2b12X1X2 + b13X1X3 + b23X2X3$$

Y is the products yields, b_0 is constant, b_1 is the linear coefficient, $b_{11}$is the quadratic coefficient, b_{12} is interaction coefficient and X_1, X_2 are the variables

RESULTS AND DISCUSSION

Screening and the microorganism.
After screening study for the seven isolated fungal strain, the best one was identified as *Absidia corymbifera*. The strain showed the ability to transform about 33% from the substrate to AD and about 12.5% to ADD the other by products were ignored (Fig.2a). The genus Absidia was described by van Tieghem in1876. It is one of 4979 genera1 belonging to thebiological kingdom of the Fungi. Members of the genusare saprobics, easily isolated from soil and decayingvegetation.*Absidia*sp is belonged to order Mucorales which is common to perform transformation of foreign material [27].

Types of transformation medium.
Seven growth media of synthetic and natural composition were used to select the most suitable one for formation of AD and ADD as shown in Fig(2 b). The lowest production of the desiredproductswas noticed when Dox's medium was used. While, the medium containing molasses and some salts was the best. The media containing high concentrations ofcarbohydrates have negative influence on the biotransformationprocess, because these types of organiccarbon source are preferred by the microorganismsover the sterols. The organic nitrogen compoundsinfluenced beneficially the process.

Duration of the biotransformation reaction.
The time periods of incubation can affect the reaction outputs. This can be seen when the time of incubation was varied (Fig 3). The optimum time appeared to be at 24 h after substrate addition. Increasing the time after 24 resulted in noticeable decrease for the desired products AD and ADD. The same effect was notice for incubation periods less than 24 h.

The substrate concentration.
The data in Fig (4) indicate that the increasing of the substrate concentration can result in decrease of products formation. This may be attributed to the toxicity of the substrate and the products to the fungal cells which can increase gradually with the gradual rising of the substrate concentration. This effect was will know and one of the inherent problem during the biotransformation processes. These have been reviewed indetail by Malaviya and Fernandes [27, 16, 28]. The isolation of new microorganisms aims to evaluate robust organisms that can withstand their ownproducts.

The Maximization steps.
Seven different cultural factors were screened for the most effective ones. The factors screening by two levels factorial design namely, (+) and (-) is indicated in table (2). The effect of seven variables were analyzed by multiple regression analysis method and indicated in Table (3). The results indicated that, molasses concentration, time and tween are the highly significant factors (<0.05). The effects of the factors on the conversion process are indicated in Fig (5) for AD and in Fig (6) for ADD. Some factors have positive effect and some others have negative effects. The Paret chart (Fig.7) showed a variation between the effects of the factors. This was noticed for each f the produced AD and ADD. According to these results the three factors namely, molasses, time and tween can be chosen for further optimization step.

Box-Behnken model was used to optimize the conditions for the most significant factors. This model is three levels design, each factor was investigated at three levels, (+), (0) and (-1) as shown in table (4). The analysis of variance (ANOVA) was carried out for the results in Table (5) for the two products, AD and ADD. The higher values of determination coefficient R^2 0.91, 0.90 for ADD and AD, respectively confirm type effectivenessof the model. The experimental data were fitted to a second order polynomial model obtained by multiple regression analysis. ANOVA was used to test the adequate of the model. The high value of R^2 indicate a good consistency between the experimental data and the data from the model. The model equations in coded term are as a follow:

$$AD= +14.45+ 1.27*A+0.17*B-3.40 *C-0.11*A*B-0.51*A*C+ 0.47*B*C-0.52*A^2- \quad 0.99*B^2-0.55*C^2.$$

$$ADD=+56.20+19.82*A+1.04*B-2.08*C+0.39*A*B-3.97*A*C-10.81*A^2+ \quad 2.64*B^2+1.90*C^2$$

The data that are represented in Fig (8) show the interaction between the three significant factors, molasses concentration, time and temperature. As shown a maximum value of AD an ADD can be achieved when the terms near 24-30h, molasses 20g/l and temperature 25-30º C.

.Table (1) Assay for the common phytosterols in plant parts of *Jatropha*sp

The common Phytosterols	Quantity (g/kg)	Percent of sterols in unsap (%).
Campsterols	0.22	3.14
Stigmasterols	0.23	3.21
β-sitosterols	2.68	38.28
Δ^5-avenatserols	0.16	2.18
Total unsap. fraction	7	

Table (2) The two levels factorial design and the results for screening of cultural factors

No.	Molasses	Y.E	Sod. citrate	K_2HPO_4	$MnSO_4$	Tween	Time	ADD	AD
1	40	4	0.01	5	0.05	0.3	24	27.33	18.11
2	40	2	0.01	3	0.05	0.1	24	19.55	16.23
3	10	2	0.01	5	0.03	0.3	24	48.13	21.44
4	10	2	0.01	3	0.05	0.3	24	37.22	22.34
5	10	4	0.01	5	0.05	0.1	24	28.16	19.67
6	10	2	0.01	5	0.05	0.1	12	25.55	16.65
7	40	4	0.01	3	0.05	0.1	12	17.19	14.65
8	10	2	0.01	3	0.03	0.1	12	15.66	12.2
9	10	4	0.01	5	0.03	0.3	12	32.34	18.99
10	40	4	0.01	3	0.03	0.3	24	35.11	16.68
11	40	2	0.01	3	0.03	0.3	12	27.23	15.76
12	10	4	0.01	3	0.05	0.3	12	25.55	16
13	40	4	0.01	5	0.03	0.1	12	19.23	13.16
14	40	2	0.01	5	0.05	0.3	12	24.61	17.89
15	10	4	0.01	3	0.03	0.1	24	32.66	18.87
16	40	2	0.01	5	0.03	0.1	24	19.56	17.88

Table (3) Statistical analysis for the Blckett-Burman design and the results

Source	Sum of Squares		df		Mean Square		F Value		p-value Prob > F	
	ADD	AD	ADD	AD	ADD	AD	ADD	AD	ADD	AD
Model	872.03	95.31	6	7	145.34	13.62	6.17	6.94	0.0082*	0.0069*
A-Molasses	192.24	15.6	1	1	192.24	15.6	8.16	7.96	0.0189*	0.0225*
C-Sod. citrate	0.59	1.13	1	1	0.59	1.13	0.025	0.58	0.8775	0.4688
D-K2HOP4	13.58	6.23	1	1	13.58	6.23	0.58	3.17	0.4672	0.1127
E-MnSO4	38.32	7.65	1	1	38.32	7.65	1.63	3.9	0.2342	0.0838
F-Tween	399.6	2.69	1	1	399.6	2.69	16.96	1.37	0.0026*	0.2752
G-Time	227.71	20.03	1	1	227.71	20.03	9.66	10.21	0.0126*	0.0127*
Residual	212.11	41.99	9	1	23.57	41.99				
Cor Total	1084.14	15.69	15	8		1.96				

Table (4) The Box-Behnken design and the obtained results for AD and ADD

No.	Molasses (g/l)	Tween (%)	Time (h)	ADD (%)	AD (%)
1	10	0.4	24	73.22	18.33
2	10	0.3	30	68.11	13.34
3	10	0.4	36	57.34	13.87
4	7.5	0.4	30	59.11	14.15
5	5	0.4	36	29.45	9.45
6	7.5	0.5	24	63.56	15.33
7	7.5	0.4	30	47.16	13.22
8	7.5	0.4	30	47.25	13.44
9	7.5	0.3	36	61.67	15.55
10	5	0.3	30	25.44	6.5
11	7.5	0.4	30	62.33	14.99
12	7.5	0.5	36	59.56	12.11
13	5	0.5	30	27.33	6.77
14	7.5	0.3	24	58.44	17.66
15	10	0.5	30	71.55	16.18
16	5	0.4	24	29.45	8.88
17	7.5	0.4	30	65.45	16.45

Table (5) Statistical analysis of Box-Behnken model for AD and ADD

Stat. measure	ADD	AD	Stat measure	ADD	AD
Std. Dev.	7.09	1.37	R-Squared	0.91	0. 90
Mean	53.32	13.48	Adj R-Squared	0.81	0.76
C.V. %	13.29	10.15	Pred R-Squared	0.66	0.14
PRESS	1389.75	109.91	Adeq Precision	8.75	9.23

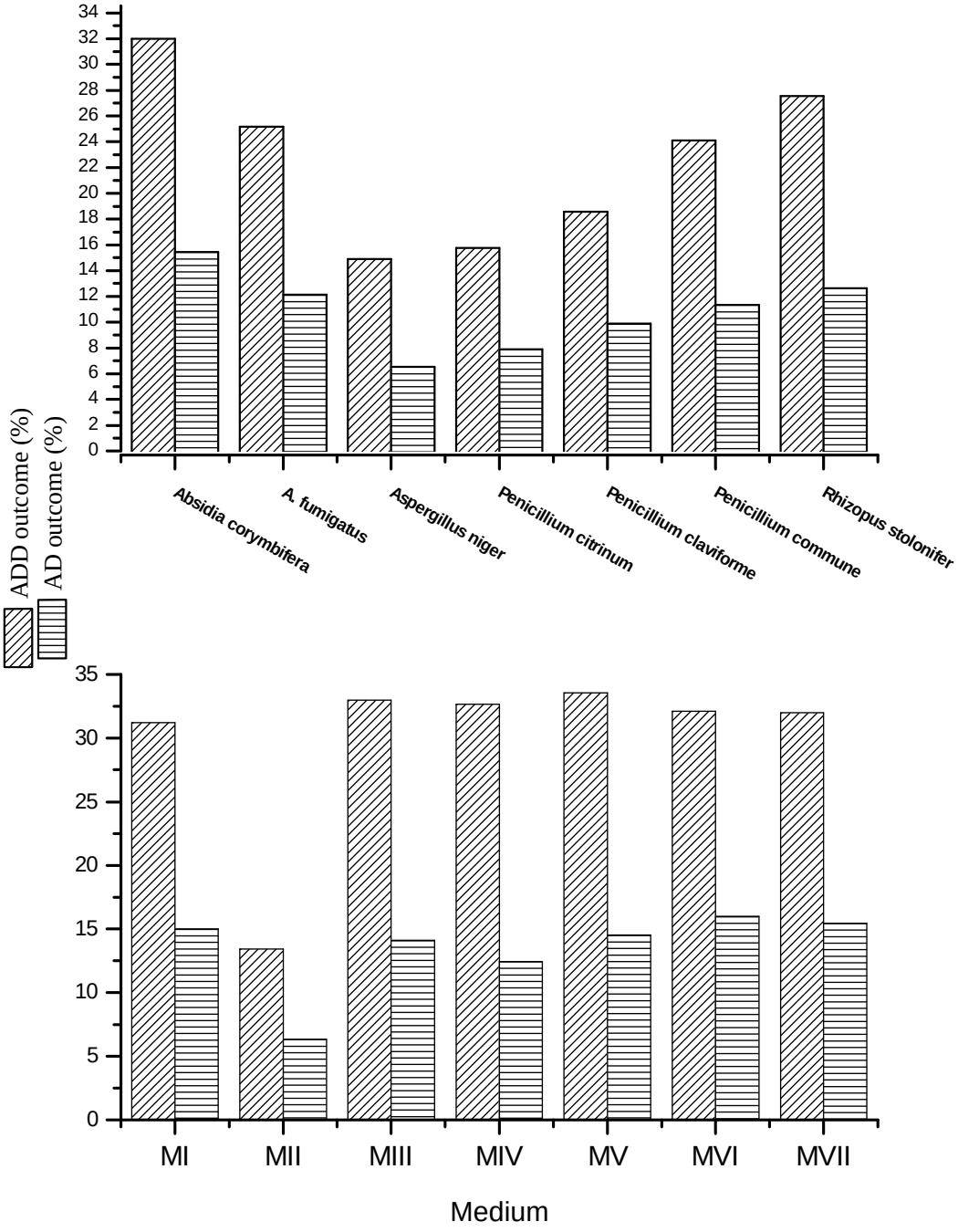


Fig. (2) Some sterols converting microorganisms and the suitable medium for the conversion process

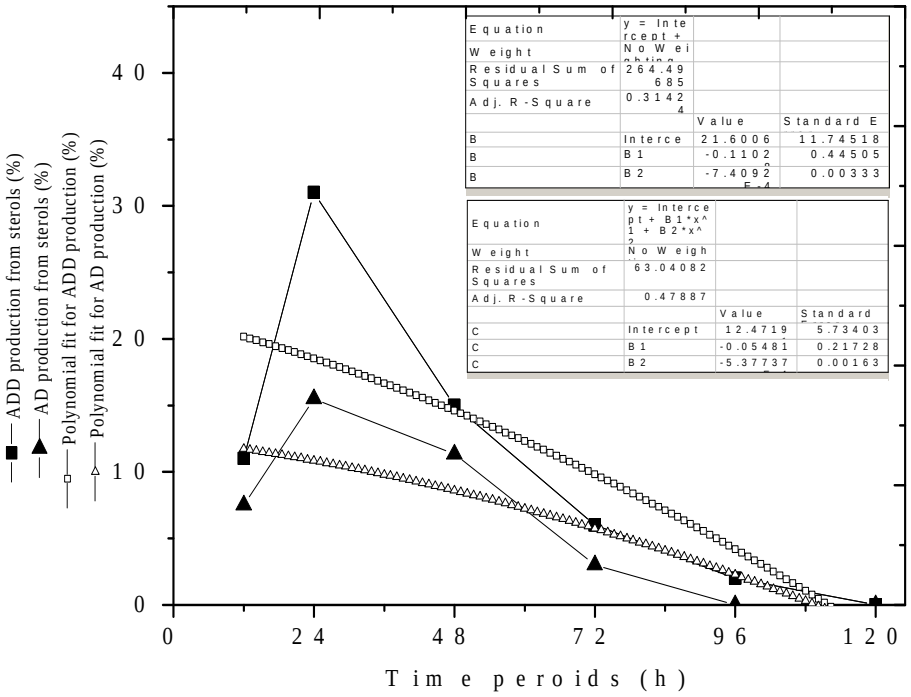


Fig (3) The optimum time for the conversion of the sterols to AD and ADD

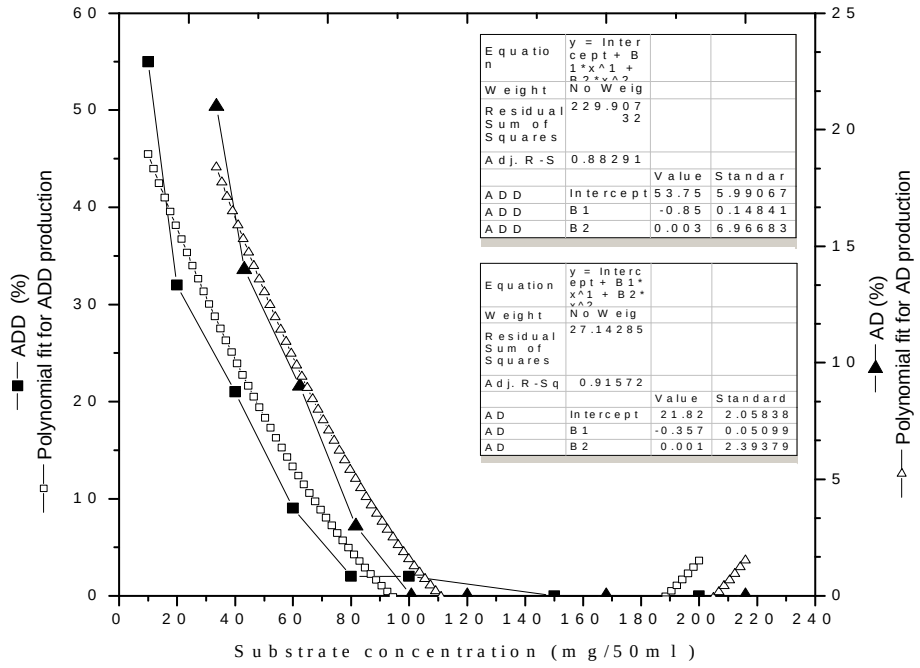


Fig (4) The ability of the tested microorganism to withstand the stress of the substrate concentrations

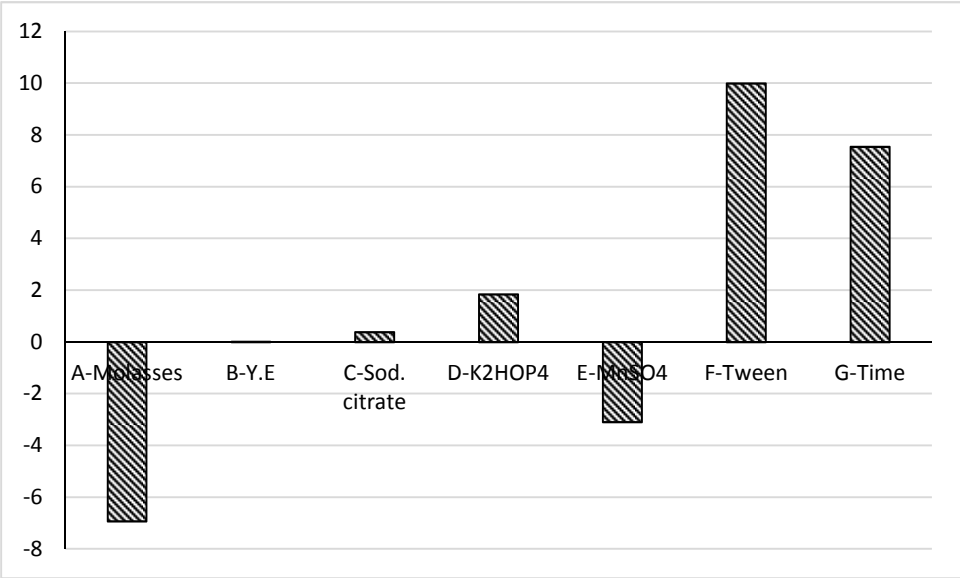


Fig (5) The effect of the factors on the conversion process of sterols to ADD

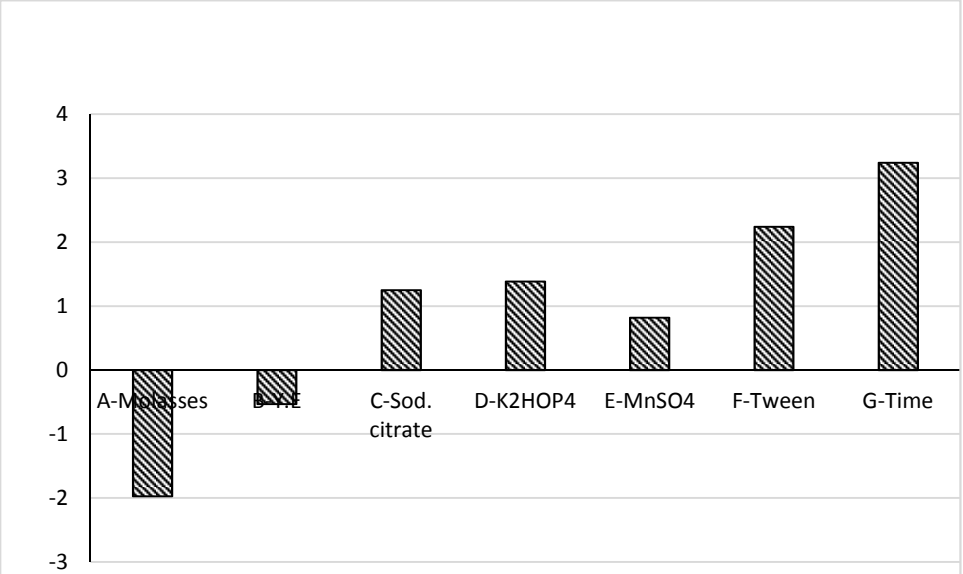
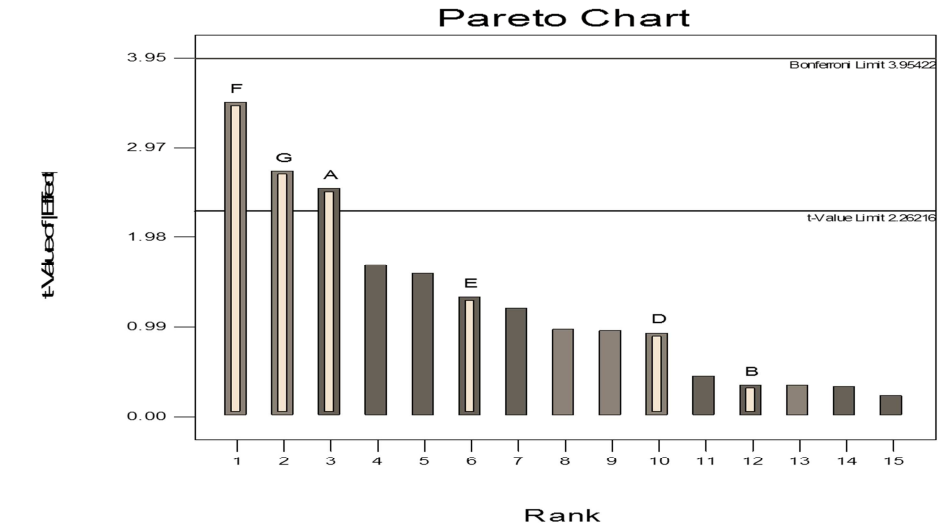
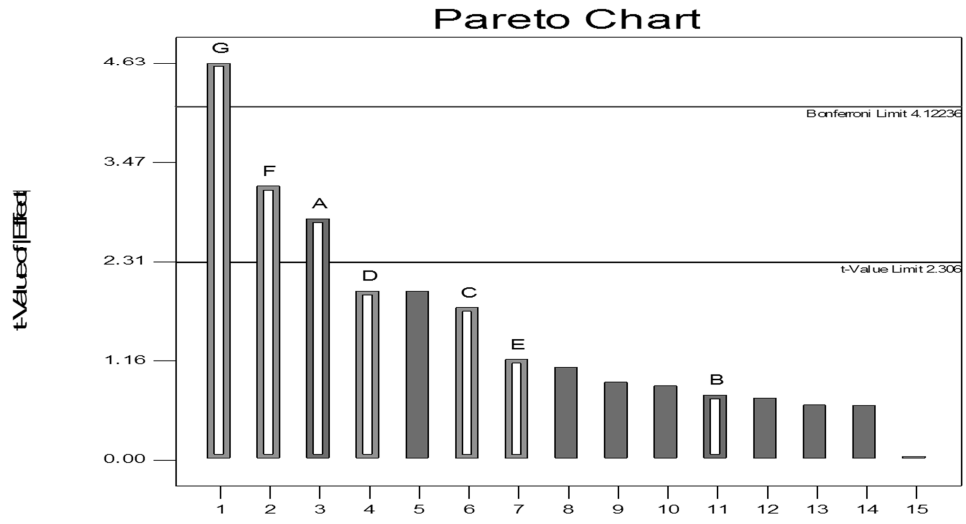


Table (6) The effect of the factors on the conversion process of sterols to AD



a



b

Fig (7) The Parto chart for some cultural factors affeting conversion of sterols to AD (a) and to ADD (b)

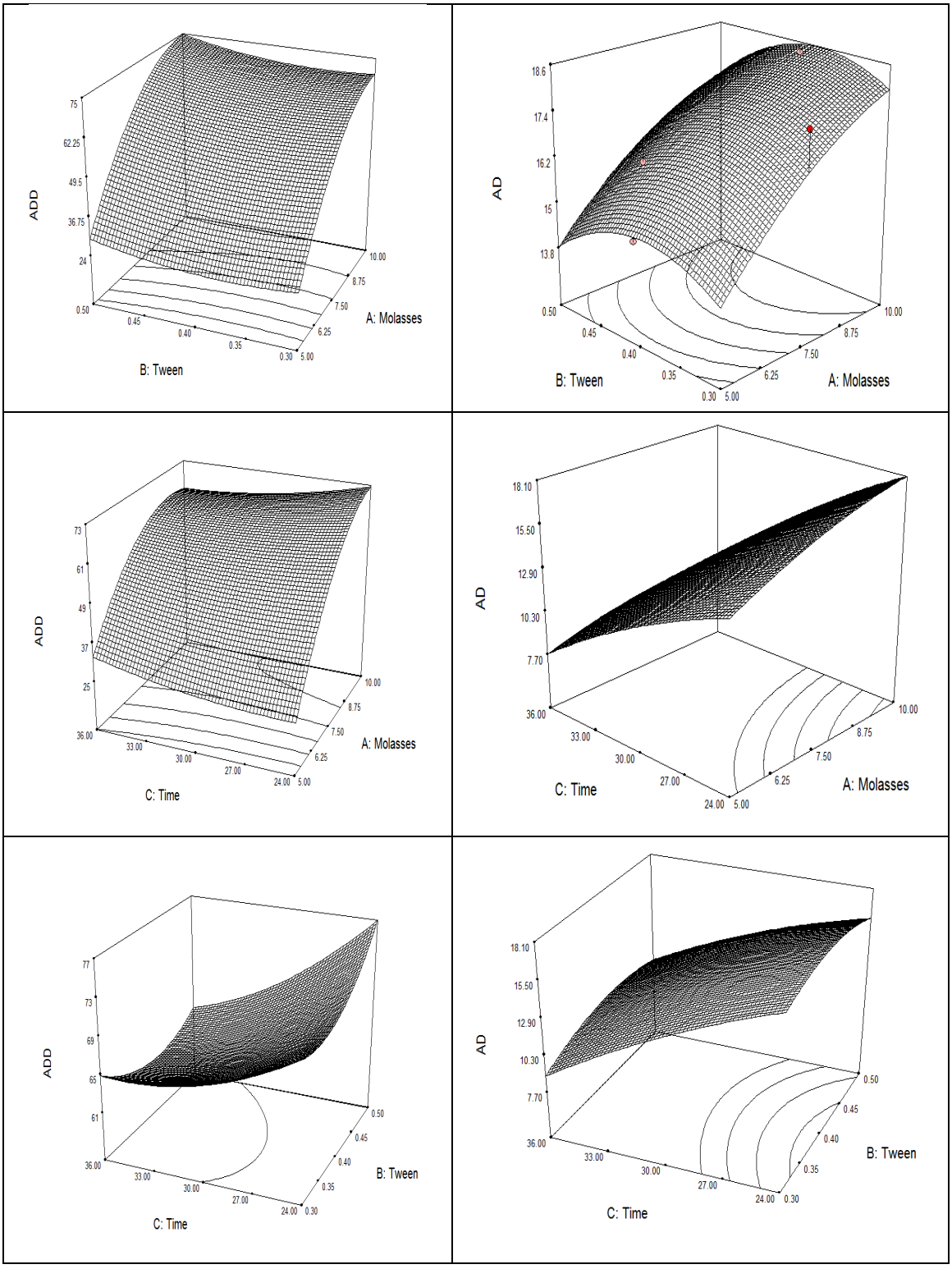


Fig (8) The interaction between the most signicant factors affecting the conversion of sterols to AD and AADD according to Box-Behnken model

CONCLUSION

The sterols extracted from all parts of *Jatropha* plant were able to provide a source for steroids, AD and ADD. The isolated *Absidia corymbifera* had the ability to withstand the metabolic stress of the biotransformation process. The optimization steps led to two fold increase in ADD formation which is the major product of the biotransformation process.

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