International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.10 No.15, pp 473-481, 2017

Validating a Mathematical Model Describing Two Antagonistic Parallel Ways for Optimum Biotransformation of β-Sitosterol to Testosterone

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Abstract : The current study aimed at optimizing microbial transformation of β-sitosterol to testosterone through statistical methods of modeling and optimization. Plackett-Burman design was employed to screen the significance of eleven factors in the biotransformation of β-sitosterol to testosterone by the strain *Rhizopus oryzae* nrc11. Ten factors were of a significance and three of them, namely yeast extract, β-sitosterol and β-carotene concentration, were the most affecting and were thus further studied through Box-Behnken design. The proposed model showed a strong interaction exerted by each of β-sitosterol and β-carotene with the role of yeast extract. Promotion of the biotransformation process could be attained by one of two ways that are antagonistic to each other and so only one of them should be applied to avoid antagonistic mechanisms. One of the two ways was attained by increasing yeast extract level while keeping β-sitosterol and β-carotene at the lowest levels. The other way was the inverse image of the previous form; increased levels of the later components while keeping the former, yeast extract, at lowest level. Both of the two ways were practically validated and the first was the most efficient as its molar conversion percent was two times higher than that of the second.
Keywords : β-sitosterol, Testosterone, Biotransformation, Plackett-Burman, Box-Behnken.

Introduction

Phytosterols are plant naturally occurring steroid alcohols [1]. Phytosterols could be afforded commercially from wastes like soybean oil deodorizer distillate [2]. β-sitosterol is one of the most abundant phytosterols in vegetables, legumes, cereals, fruits and vegetable oils [3]. Nevertheless, other types of phytosterols like stigmasterol, campesterol and brassicasterol are present also but in smaller amounts. In some wheat grain tissues, β-sitosterol comprised around 60% of all phytosterols content [4].

In the human body, there are three categories of endocrine hormones belonging to the steroid class namely corticosteroids, sex steroids, and anabolic steroids [1]. Testosterone is one of sex hormone that plays many roles in male and female bodies. It acts as the basic operator for male sex characteristics in males.

For synthesis of steroidal hormones, biotransformation of phytosterols is the process of choice due to having the advantages of being simple, environmental friendly and of low costs. Although many endeavors have been dedicated for biotransformation of phytosterols to steroidal hormones or their precursors, very scarce activities were directed to optimize biotransformation process parameters using statistical methods of design and modeling [5-11].

The strategies employed to improve fermentation medium are very numerous [12]. It was thought that classical approach of one-at-a-time investigation has the disadvantage of being tedious and time consuming. The worst disadvantage is the limited gain of optimization comparing with statistical design [12]. This simply returns to the fact that classical one-at-a-time strategy neglects completely interactions between variables. On the other side, the superior advantage of some statistical designs is the ability to clarify interactions between factors and to identify many optima from which designer could select basing on his economic point of view. As such, optimization the process of conversion of the cheap substrate, β-sitosterol, to a valuable end product, testosterone, via statistical methods of design and modeling was chosen to be the aim of the current study.

2. Materials and Methods

2.1. Organism

Rhizopus oryzae nrc11 was kindly provided from the Natural and Microbial Products Chemistry Department, National Research Centre (NRC), Dokki, Giza, Egypt. Culture was maintained on potato dextrose agar slant and sub-cultured regularly on the same medium.

2.2. Chemicals and solvents

β-sitosterol, authentic testosterone, β-carotene and ascorbic acid were purchased from Sigma Chemicals Company, USA. Solvents of analytical grade, yeast extract, meat extract, glucose, corn steep liquor and cane molasses were purchased locally.

2.3. Fermentation

Fermentation was carried out in Erlenmeyer flasks (250 ml) containing 50 ml of fermentation media. Flasks were inoculated with 0.5 ml/flask of spore suspension (containing 10^4-10^5 spore/ml) of *Rhizopus oryzae* nrc11. Immediately after inoculation, 0.5 ml of β-sitosterol solution (10 g/l) in absolute ethanol was added to each flask under sterile condition to induce enzymes involved in β-sitosterol metabolism. Then flasks were incubated in shaking incubator at 30ºC±2 and 150 rpm in all experiments till mention otherwise. After 24 hrs, the main amount of β-sitosterol substrate for biotransformation was added as 2.5 ml/flask of the substrate solution (10 g/l) in absolute ethanol to get the final concentration of 25 mg/flask of β-sitosterol. After that, flasks were retuned to incubator (30ºC±2 and 150rpm) to be allowed for biotransformation along two days. Fermentations was done in duplicate flasks for each treatment and after separate determinations for each, arithmetic average was calculated to express the result for the treatment.

2.4. Extraction

At the end of incubation period of biotransformation (2 days), contents of each flask were extracted by shaking with the same volume of chloroform for 30 minutes after which the mixture was allowed to stand and the solvent layer was separated, air dried and re-dissolved in 2 ml of chloroform.

2.5. Separation

Ten microliters of chloroform final extract were applied onto thin layer chromatography (TLC) silica gel aluminum sheets (20x20, 0.25 mm, F254, Merck) and allowed to developed in dichloromethane: petroleum ether: ethyl acetate (6:3:1, v/v/v) solvent system [13]. Under ultraviolet illumination and comparing with authentic sample, spots of produced testosterone were visualized and marked thoroughly by pencil to be latter scratched, collected in small test tube for each sample and assayed quantitatively for testosterone. In case of β-sitosterol, spots needed to be sprayed with Liebermann-Burchard reagent [14] that was prepared as follow: 5 ml sulfuric acid were added drop-wise to 5 ml acetic anhydride then the mixture was added drop-wise to cooled 50 ml absolute ethanol. After spraying authentic β-sitosterol sample, plates were heated at 110 ºC for 10–15 min in an oven to develop a color that is seen in day light. Comparing with authentic spot, β-sitosterol in plates could be marked to be later scratched and assayed quantitatively.

2.6. Quantitative determination of testosterone

Testosterone in each sample after being separated as a spot on TLC plate, was scratched and transferred to small test tube (10 ml) where 4 ml of chromogen reagent were added. Chromogen reagent was prepared by dropwise addition of 45 ml concentrated sulfuric acid to cooled 55 ml of absolute ethyl alcohol [14]. The mixture of sample and chromogen was dispersed well in a vortex and boiled for 20 minutes in a water path. The mixture was then cooled, centrifuged at 3000 rpm and absorbance of supernatant was measured at 460 nm in UV-Vis Jasco spectrophotometer. By the same way, standard concentrations of testosterone were assayed and plotting the relation between concentration and absorbance showed straight line whose linear equation was used to get testosterone concentrations from absorbance readings of samples.

2.7. Statistical design and modeling

Statistical designs (Plackett-Burman and Box-Behnken), analysis of variance (ANOVA) of data, regression analysis to get polynomial coefficients and equations, three-dimensional response surface plots and predictions of the optimum levels of variables were achieved using the 'Design Expert' software (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA) statistical package.

3. Results and Discussion

3.1. Plackett-Burman design

Plackett-Burman design was used as a simple tool to screen large number of variables with low number of runs to finally detect factors of importance in the process. It assumes the linear approach is considered to be sufficient for screening. General equation of the linear relation is simplified as follow:

$$Y = \beta_0 + \sum \beta_i X_i \quad (i = 1, \ldots, k)$$

Where Y is the target function; β_i is the regression coefficient of factor X_i; β_0 is the model intercept. Surveying literature about biotransformation of sterols showed that many nutritional agents could be involved in the formulation of culture media [7, 15, 16). The most common nutrients recorded in the literature about the conversion of sterols were tested in the current study for their effect in the conversion of β-sitosterol to testosterone by Plackett-Burman design (Table 1). In addition, the effect of some vitamins (ascorbic acid, vitamin C, and the precursor of vitamin A, β-carotene) playing a known role of antioxidant activities, as well as β-sitosterol concentration and time course of fermentation were also studied. Eleven variables were screened at high (+1) and low (-1) levels and one central point (0). Table 1 showed specifications of the variables, codes and levels.

Table 1: Factors and levels studied by Plackett-Burman.

Factor code	Name (Units)	Low level (−1)	Mean level (0)	High level (+1)	Std. Dev.
A	Peptone concentration (g/l)	3	5	7	1.922
B	NaCl concentration (g/l)	0	2.5	5	2.402
C	Yeast ext. concentration (g/l)	1	2	3	0.961
D	Meat ext concentration (g/l)	0.5	1	1.5	0.48
E	Glucose concentration (g/l)	0	2.5	5	2.402
F	Corn steep concentration (%v/v)	0.5	1	1.5	0.48
G	Molasses concentration (g/l)	10	20	30	9.608
H	β.sitosterol concentration (g/l)	0.3	0.5	0.7	0.192
J	Ascorbic acid concentration (mmole/l)	0	0.5	1	0.48
K	β.carotene concentration(mmole/l)	0	0.05	0.1	0.048
L	Time course (day)	1	2	3	0.961

The simplicity of Plackett-Burman design provided the opportunity to screen the effects of eleven components (peptone, NaCl, yeast extract, meat extract, glucose, corn steep liquor, cane molasses, ascorbic acid, β-carotene, β-sitosterol concentration and time course of fermentation). It was not feasible to study all

these variables with the conventional one-at-a-time development strategies. Table 2 showed the design, which consisted of only 13 runs, with corresponding responses. These data were statistically analyzed by ANOVA to get the outputs given in Table 3. The model F-value of 4542.24 implies the model is significant. There is only a 1.15% chance that a "Model F-Value" could occur due to noise.

Table 2: Plackett-Burman experimental design for eleven factors affecting conversion of β-sitosterol to testosterone by *Rhizopus oryzae nrc11*

	Factors*											Testosterone yield (µg/flask)
Run	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**J**	**K**	**L**	
1	1	-1	-1	-1	1	-1	1	1	-1	1	1	282.298
2	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	100
3	-1	1	1	1	-1	-1	-1	1	-1	1	1	306.832
4	-1	1	1	-1	1	1	1	-1	-1	-1	1	225.155
5	1	-1	1	1	-1	1	1	1	-1	-1	-1	245.652
6	1	1	-1	-1	-1	1	-1	1	1	-1	1	218.323
7	-1	-1	1	-1	1	1	-1	1	1	1	-1	342.547
8	0	0	0	0	0	0	0	0	0	0	0	286.646
9	1	1	-1	1	1	1	-1	-1	-1	1	-1	191.615
10	-1	1	-1	1	1	-1	1	1	1	-1	-1	282.298
11	1	-1	1	1	1	-1	-1	-1	1	-1	1	268.634
12	1	1	1	-1	-1	-1	1	-1	1	1	-1	298.758
13	-1	-1	-1	1	-1	1	1	-1	1	1	1	245.031

* Factors were coded and applied in unites and levels (-1, 0, +1) that are indicated in Table 1.

Table 3: Statistical analysis of Plackett-Burman model

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value
Model	44173.53047	10	4417.353	4542.236	0.0115
B-NaCl conc.	125.5818963	1	125.5819	129.1322	0.0559
C-Yeast ext. conc.	11286.09525	1	11286.1	11605.17	0.0059
D-Meat ext conc.	443.8566542	1	443.8567	456.405	0.0298
E-Glucose conc.	2638.859419	1	2638.859	2713.463	0.0122
F-Corn steep conc.	414.1510101	1	414.151	425.8595	0.0308
G-Molasses conc.	1906.184496	1	1906.184	1960.074	0.0144
H- β.sitosterol conc.	10135.99816	1	10136	10422.55	0.0062
J-Ascorbic acid conc.	7703.221648	1	7703.222	7921	0.0072
K-B.carotene con.	8911.765557	1	8911.766	9163.711	0.0067
L-Time course	607.816378	1	607.8164	625	0.0255
Curvature	1199.681305	1	1199.681	1233.598	0.0181
Residual	0.972506205	1	0.972506		
Cor Total	45374.18428	12			
R-Squared (Correlation coefficient) 1.00		Adjusted R-Squared 0.9998			
Adequate Precision 255.994					

Model terms having a p-value less than 0.05 were significant terms. Accordingly, all studied variables, except peptone concentration, were significant variables. The most affecting variable was yeast extract concentration (p-value 0.0059) followed by β-sitosterol (p-value 0.0062) and β-carotene (p-value 0.0067) concentrations. The "Curvature F-value" of 1233.60 implied there was a significant curvature (as measured by the difference between the average of the center points and the average of the factorial points) in the design space. There is only a 1.81% chance that a "Curvature F-value" could occur due to noise. "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. "Adequate Precision" of the design was 255.994 which indicated an adequate signal. As such, this model can be used to navigate the design space and the best equation describing the model was the following:

Testosterone concentration (µg/flask) = 6.09472 + 1.29400 B + 30.66770 C + 12.16356 D + 5.93168 E – 11.74948 F + 1.26035 G + 145.31573 H + 50.67288 J + 545.03106 K + 7.11698 L

Where B, C, D, E, F, G, H, J, K and L are the codes of studied factors as indicated in Table 1. However, care should be taken when applying this design outside screening target because it neglects interactions between the different variables under consideration [17]. Therefore, the three most important variables (yeast extract, β-sitosterol and β-carotene concentrations) were then further investigated for design and modeling in second stage statistical factorial design, Box-Behnken design.

3.2. Box-Behnken design

Using Box-Behnken design with three center points, the effect of most important factors namely yeast extract (X1), β-sitosterol (X2) and β-carotene (X3) concentrations were studied while other factors were maintained at mean levels. This design facilitates the opportunity to clarify the fine interactions between factors. Table 4 showed Box-Behnken design of 15 run and testosterone yield for each run. The data were then analyzed by ANOVA for two-factor interaction (2FI) and outputs have been illustrated in Table 5. The best fit model describing the effect of the three studied factors was found to be first order polynomial with two-factor interaction (2FI) equation of the following general form

Table 4: Box-Behnken design for studying the most important factors affecting conversion of β-sitosterol to testosterone by *Rhizopus oryzae nrc11*

Run	Factor 1 X_1 Yeast ext. conc. (g/l)	Factor 2 X_2 β.sitosterol conc. (g/l)	Factor 3 X_3 β.carotene conc. (mmole/l)	Response (Testosterone yield µg/flask)
1	3	0.5	0.1	293.478
2	1	0.5	0.1	195.652
3	1	0.7	0.05	350.932
4	2	0.5	0.05	248.758
5	3	0.5	0	386.957
6	3	0.3	0.05	379.814
7	2	0.7	0.1	354.969
8	1	0.3	0.05	189.441
9	2	0.7	0	259.627
10	2	0.3	0	257.764
11	2	0.5	0.05	180.124
12	2	0.3	0.1	168.323
13	1	0.5	0	150.621
14	2	0.5	0.05	237.267
15	3	0.7	0.05	270.807

$Y = \beta_0 + \sum \beta i Xi + \sum \beta ij XiXj$

Where Y is the predicted response; Xi, Xj are input variables which influence the response Y; β_0 is the offset term; βi is the linear coefficient of factor Xi; βij is the coefficient of interaction between variables Xi and Xj.

Table 5: ANOVA for response surface 2FI model

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value (Prob > F)
Model	63798.05	6	10633.01	3.92	0.0395
X_1-Yeast ext. conc.	24687.52	1	24687.52	9.11	0.0166
X_2-β.sitosterol conc.	7259.75	1	7259.75	2.68	0.1403
X_3-β.carotene conc.	226.28	1	226.28	0.08	0.7799
X_1X_2	18292.14	1	18292.14	6.75	0.0317
X_1X_3	4796.21	1	4796.21	1.77	0.2200
X_2X_3	8536.15	1	8536.15	3.15	0.1138
Residual	21676.44	8	2709.55		
Lack of Fit	18973.80	6	3162.30	2.34	0.3293
Pure Error	2702.63	2	1351.32		
Cor Total	85474.49	14			
Std. Dev. 52.05		R-Squared (Correlation coefficient) 0.7464			
Mean 261.64		Adjusted R-Squared 0.5562			
C.V. % 19.9		Predicted R-Square 0.0113			
Press 84507.52		Adequate Precision 6.997			

As indicated in Table 5, the model F-value of 3.92 implied the model is significant. There is only a 3.95% chance that a "Model F-Value" could occur due to noise. The Lack of Fit" F-value of 2.34 implied the "Lack of Fit" is not significant relative to the pure error which supported the significance of the model. "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The obtained ratio of 6.997 indicated an adequate signal. Accordingly, this model can be used to navigate the design space and the final equation describing the model in terms of studied factors was represented by the following:

Testosterone concentration (µg/flask) = $-211.34576+259.23913X_1+595.88509X_2-1031.05590X_3-338.12112X_1X_2-692.54658X_1X_3+4619.56522X_2X_3$

Where X_1, X_2 and X_3 are the codes of the studied variables as indicated in Table 4.

Figure 1 depicted the three-dimensional surface plot for the response (testosterone concentration) in relative to the combination of two of the studied factors. Fig. 1a revealed a strong interaction between variables namely yeast extract and β-sitosterol concentrations; the effect of each variable may be positive or negative according to the concentration of the other variable. As shown in Fig. 1a, when maintaining β-sitosterol at low amount (0.3 g/l), increase in yeast extract was accompanied by increase in testosterone yield which indicated a positive role of yeast extract. On the other side, when maintaining β-sitosterol at high amount (0.7 g/l), increase in yeast extract was accompanied by decrease in testosterone yield indicating a negative effect of yeast extract on the biotransformation process. Similarly, the effect of β-sitosterol was positive or negative according to yeast extract concentration. Rising concentration of β-sitosterol was of a positive effect if yeast extract was maintained at low level and vice versa. As such, highest yields of testosterone have been achieved by elevating the concentration of one of the key nutrients, yeast extract or β-sitosterol, while keeping the other at low level. Getting high yields of testosterone via elevated amount of yeast with reduced level of β-sitosterol provided the economic privilege of saving the biotransformation substrate, β-sitosterol and thus achieving higher value of the conversion ratio, product/substrate.

By the same manner, Fig. 1b showed the interaction between yeast extract and β-carotene. At the low amount of yeast extract (1 g/l), increase in β-carotene was of a positive effect (resulted in an increase in testosterone yield) while at high yeast extract amount (3 g/l), the increase in β-carotene was of a negative effect. As such, it was assumed that yeast extract when applied in high amount could afford additional nutritive role which is the same as the role of β-carotene (mostly as an antioxidant factor) and thus the addition of β-carotene under such condition (high yeast extract concentration) would be over need and so exerted negative action on testosterone yield.

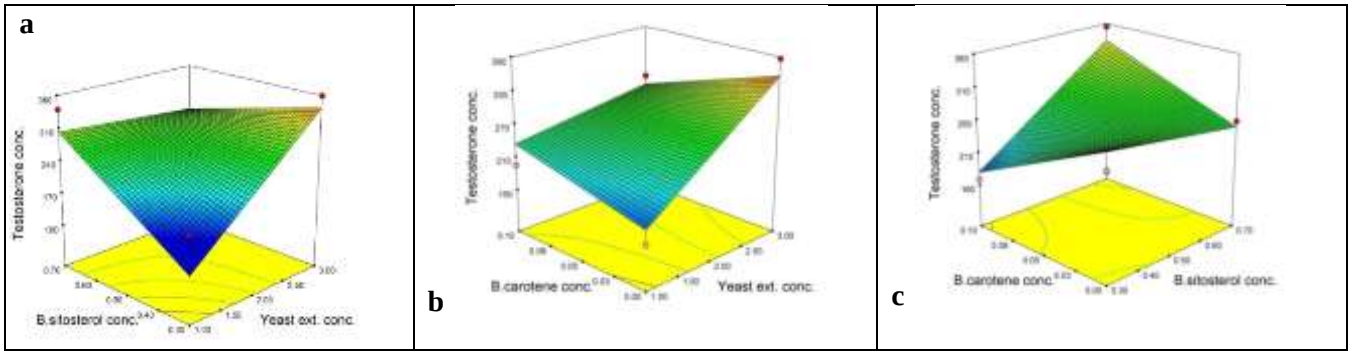

Fig 1: Three-dimensional surface plot for the relation of testosterone conc. (µg/flask) with interaction between two variables, a: yeast extract and β-sitosterol concentrations (g/l); b: yeast extract (g/l) and β-carotene (mmole/l) concentrations; c: β-sitosterol (g/l) and β-carotene (mmole/l) concentrations.

3.3. Validation of the model obtained from ANOVA analysis of Box-Behnken design

For the final equation describing the model, several solutions were obtained for highest response yield. Two of these solutions were chosen for practical validation. Level of each variable in these two media was demonstrated in Table 6. For verification, the two media were prepared and applied in the conversion process. Actual testosterone yield was determined and compared to yield predicted according to the model equation and the ratio between them in percent was expressed as the validity. Table 6 summarized all of these data as well as molar conversion percent in each medium. The two medium achieved the same testosterone yield while the

validity was 83% and 91% for the first and the second medium respectively. The composition of the first medium incredibly contained β-sitosterol content that was reduced to less than half of that in the second medium while testosterone yield was nearly the same in both media. Therefore, more than two-fold increase in molar conversion percent has been achieved in the first medium compared with the second, which strongly highlighted the economic value of the first medium and suitability for large-scale application studies. As concluded from the preceding evaluation of media, there were two parallel strategies for getting the optimal conversion process; the first was achieved by high yeast extract concentration with reduced concentrations of β-sitosterol and β-carotene (first medium) and the second was achieved by higher amounts of β-sitosterol and β-carotene with a reduced concentration of yeast extract. According to the model equation, applying the two strategies at once exerts deterioration in testosterone yield which reflects antagonistic pattern between the two strategies.

Table 6: Practical validation of two fermentation media predicted by Box-Behnken model

Medium	Variable level			Actual testosterone (µg/flask)*	Predicted testosterone (µg/flask)	Validity (Actual / Predicted)	Molar conversion** %
	Yeast extract conc. (g/l)	β-sitosterol conc. (g/l)	β-carotene conc.(mmole/l)				
1st	3	0.3	0	332.3 ± 26.4	402	83%	3.2
2nd	1	0.7	0.1	330.7 ± 11.9	364	91%	1.4
* Mean ± standard deviation. ** mmole testosterone produced per flask / mmole initial β-sitosterol per flask x100							

4. Conclusion

With the aid of Plackett-Burman design, the current study screened the effect of many factors on conversion of β-sitosterol to testosterone and determined the most potent ones. Also, the study employed Box-Behnken design to understand the roles and interactions between factors of high impact on conversion process and also to predict the conditions for optimal conversion process. Practical verification of the model proved the validity of two parallel ways to achieve highest yield of testosterone with apparent antagonism between the two ways.

Acknowledgement

Grateful acknowledgment to National Research Centre, Giza, Egypt for the financial support of the current study as a part of the project P100215 included in the extension of 10th research plan grants.

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