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Single step biotransformation of corn oil phytosterols to boldenone by a newly isolated *Pseudomonas aeruginosa*

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ABSTRACT

A new potent *Pseudomonas aeruginosa* isolate capable for biotransformation of corn oil phytosterol (PS) to 4-androstene-3, 17-dione (AD), testosterone (T) and boldenone (BOL) was identified by phenotypic analysis and 16S rRNA gene sequencing. Sequential statistical strategy was used to optimize the biotransformation process mainly concerning BOL using Factorial design and response surface methodology (RSM). The production of BOL in single step microbial biotransformation from corn oil phytosterols by *P. aeruginosa* was not previously reported. Results showed that the pH concentration of the medium, $(NH_4)_2SO_4$ and KH_2PO_4 were the most significant factors affecting BOL production. By analyzing the statistical model of three-dimensional surface plot, BOL production increased from 36.8% to 42.4% after the first step of optimization, and the overall biotransformation increased to 51.9%. After applying the second step of the sequential statistical strategy BOL production increased to 53.6%, and the overall biotransformation increased to 91.9% using the following optimized medium composition (g/l distilled water) $(NH_4)_2SO_4$, 2; KH_2PO_4, 4; Na_2HPO_4. 1; $MgSO_4{\cdot}7H_2O$, 0.3; NaCl, 0.1; $CaCl_2{\cdot}2H_2O$, 0.1; $FeSO_4{\cdot}7H_2O$, 0.001; ammonium acetate 0.001; Tween 80, 0.05%; corn oil 0.5%; 8-hydroxyquinoline 0.016; pH 8; 200 rpm agitation speed and incubation time 36 h at 30 °C. Validation experiments proved the adequacy and accuracy of model, and the results showed the predicted value agreed well with the experimental values.

1. Introduction

In pharmaceutical industries, there are an urgent need for newer and cheaper raw materials containing natural sterols for the production of steroidal compounds. Several microbial bioconversion reactions of steroids and sterols have been reported, focusing mainly on steroid hydroxylation, 1-dehydrogenation and sterol side-chain cleavage. The side- chain cleavage of sterols molecules is one of the most widely reported biotransformation reactions of steroids, several species of the genera *Arthrobacter, Bacillus*,*Mycobacterium*, *Corynebacterium*, *Mucor*, *Penicillium*, *Nocardia*, *Brevibacterium*, *Serrattia and Protoaminobacter*, were reported for production of steroidal derivatives by side- chain cleavage of sterols.

They have a wide application as progestational agents, diuretics, anabolic and contraceptive agents, also as replacement agents in the treatment of adrenal insufficiencies, and recently steroidal glycoside exhibited anti-viral activity of herpes virus type I [1]. AD which is a naturally occurring steroidal hormone produced in the gonad or ovary, is an immediate precursor of testosterone in males and estradiol and estrone in females. Testosterone and dihydrotestosterone are the most potent male sex hormones [2]. Boldenone (BOL) is androgenic-anabolic steroid (AAS); which increases nitrogen retention, protein synthesis, appetite and stimulates the release of erythropoietin in the kidneys. Boldenone undecylenate is often prescribed for patients that have lost muscle mass due to extended periods of bed rest or bouts with significantly diminished muscle mass. In addition, Boldenone is a popular drug for administration in various animals and extensively used in the cattle and meat production industry.

From biochemical analysis and 16S rRNA sequencing, five different bacterial isolates capable of transforming corn oil phytosterols were isolated and identified as *Pseudomonads aeruginosa* (four strains) and one as *Alcaligenes aquatilis*. The application of response surface methodology RSM aims to rapid screening of experimental factors and ingredients interactions; it resulted in the determination of the optimal process parameters

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Table 1
Biochemical properties of the isolates.

Test		Isolate				
		W	A	B1	B2	B3
Gram stain		−ve rods	−ve rods	−ve rods	−ve rods	−ve rods
Growth on mannitol salt agar		−ve	−ve	−ve	−ve	−ve
Growth on macconkey agar		+ve	+ve	+ve	+ve	+ve
Lactose fermentation on macconkey agar		−ve (yellow)	+ve (red)	−ve (yellow)	−ve (gry)	−ve (green)
Growth on cetrimide agar		+ve (yellow)	−ve	+ve (yellow)	+ve (green)	+ve (green)
Biofilm formation on congo red		+ve (black)	+ve (black)	+ve (black)	+ve (black)	+ve (black)
DNA agar (DNAse test)		−ve	+ve	−ve	−ve	−ve
Catalase test		+	+	+	+	+
Oxidase test		+	+	+	+	+
Urease		+	+	+	+	+
Gelatinase		+	–	+	+	+
Blood hemolysis		A	γ	β	β	β
MIO medium	Indol production	−ve	−ve	−ve	−ve	−ve
	Motility	+ve	+ve	+ve	+ve	+ve
	Ornithine decarboxylase	+ve	−ve	+ve	+ve	+ve

that significantly brings down the time and monetary value of experimentation [3]. Traditional optimization of fermentation factors (one at a time) is generally a time consuming and labor-intensive process. Moreover, RSM has an efficient mathematical approach based on the underlying rules of statistics such as randomization, replication and duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner widely applied in the optimization of fermentation [4].

2. Materials and methods

2.1. Chemicals

The authentic steroids used; β-sitosterol 99%, campesterol 98%, stigmasterol 99%, androst-4-ene 3,17-dione 98%, testosterone 99% and boldenone 99% were purchased from Sigma chemical company (USA). Tryptone, beef extract, bacterial peptone and nutrient broth were purchased from Difco (Germany). TLC Al pre-coated silica gel G UV (F254), disodium hydrogen phosphate, sodium sulphate (anhydrous), yeast extract and ammonium sulfate were from Merck 8-hydroxyquinoline from Riedel-deHaen. Tween 80 from VWR (Germany). Crude corn oil used in the current work was obtained from National Company for maize products, Egypt. All other chemicals and solvents used were of analytical grade and were held from various standard sources.

2.2. Isolation of microorganisms

Samples of soil were collected from factories producing corn oil. Enrichment culture technique [5] was applied, corn oil as a sole carbon and energy source was used to isolate bacterial strains. Soil samples were suspended in distilled water, shaked and cultured in selective isolation medium [6] containing (g/l): agar, 20; NH_4NO_3 17; K_2HPO_4, 0.25; $MgSO_4$. H_2O, 0.25; $FeSO_4$. H_2O, 0.001; NaCl, 0.005; Tween 80, 0.1%; corn oil 1%; fluconazole 150 mg as antifungal. The plates were incubated at 30 °C for 5 days at pH 7. Only microorganisms that can utilize corn oil as a sole carbon and energy source will grow and then isolated, purified and transferred to maintenance slants. Five bacterial strains were isolated and maintained on modified nutrient agar [7] at pH 6.8–7.2. The agar slopes were incubated for 24 h at 30 °C then stored at 4 °C with monthly subculture maintenance. The isolated strains were assigned the following abbreviations A, W, B1, B2 and B3 and were identified by biochemical activity and by 16S ribosomal RNA (rRNA) gene sequencing analysis.

2.3. Phenotypic and genotypic characterization

2.3.1. Phenotypic characterization

Gram staining and biochemical tests (oxidase, urease, catalase, DNAse, indole production and citrate utilization) were accomplished for identification of the five isolates as well as growth on different selective (mannitol salt agar and cetrimide agar) and moderately selective (macconkey agar) media (Table 1).

2.3.2. 16rRNA gene sequencing and analysis

DNA extracted by Wizard® Genomic DNA purification kit supplied by Promega (Promega, Southampton, UK) according to the manufacturer's guidelines. PCR amplification was done in 50 μl reaction volumes containing 2 μl of each primer, 8 μl of master mix, 2 μl of genomic DNA, 36 μl sterile distilled water. Amplification was done using the following profile; initial denaturation for 5 min at 95 °C, 30 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 52 °C, and extension for 1 min at 72 °C, and final extension for 10 min at 72 °C. Amplified products were detected by agarose gel electrophoresis in 1% Tris-Acetate-EDTA (TAE). Agarose gel stained with ethedium bromide and visualized on an ultraviolet illuminator. The following two primer pairs (sequence 5′-3′) were used for amplification of 16 S rRNA gene:

27F AGA GTT TGA TCM TGG CTC AG
1492R CGG TTA CCT TGT TAC GAC TT

DNA was sequenced using an automated sequencing system (BGI-Beijing, China). The amplified sequences were aligned to the sequences from the NCBI database. 16 S rRNA gene sequences were aligned with the 16 S sequences available in the NCBI database using the BLAST server (http://blast.ncbi.nlm.nih.gov/). 16 S sequence of the highly matched strains was then aligned with our assembled whole contig (1537 bp) using CLUSTAL W (www.ebi.ac.uk/tools/msa/clustalw2/).

2.4. Determination of corn oil phytosterols profile by gas chromatography

Gas chromatography (Hewlett Packard, 6890) equipped with a flame ionization detector was used. Oven with initial temperature 180 °C and maximum 300 °C at initial time 0 min, equilibration time 3 min and run time 40 min and a capillary column (model number; HP 19091Z-413 HP1-methyl siloxane) with a maximum temperature 325 °C, nominal length: 30 m, nominal diameter: 320.00 μm, nominal film thickness 0.25 μm. The mode was constant flow 2 ml/min, inlet pressure 14.83 Pa; average velocity 41 cm/s and back inlet mode was splitless at initial temperature

275 °C. Detector temperature 295 °C as the hydrogen flow was 30 ml/min and air flow was 350 ml/min. Helium was the carrier gas at a flow rate of 30 ml/min [7].

2.5. Screening the ability of the five isolates for phytosterols biotransformation

Using growing cells a screening experiment was performed in two consecutive steps, namely, activation culture in activation medium and biotransformation culture in production medium. Firstly the bacteria were activated for phytosterols biotransformation by growing aerobically at 30 °C ± 2, 200 rpm for 24 h in 250 ml Erlenmeyer flasks, each containing 100 ml portion activation medium of the following composition (g/l distilled water): $(NH_4)_2SO_4$, 2; KH_2PO_4, 4; Na_2HPO_4. 0.1; $MgSO_4{\cdot}7H_2O$, 0.3; NaCl, 0.1; $CaCl_2{\cdot}2H_2O$, 0.1; $FeSO_4{\cdot}7H_2O$, 0.001; ammonium acetate 0.001; Tween 80, 0.1%; corn oil 1%; 8-hydroxyquinoline 0.016 and pH adjusted to 7. After activation step; 2 ml bacterial broth used for inoculation of three different production media [8–10]. The media were autoclaved at 121 °C (15 lb/inch^2) for 15 min 2 ml culture was transferred to a 250 ml flask containing 50 ml of the production medium, the flasks were incubated at 30 °C ± 2, 200 rpm for 120 h. Isolate with highest yields was selected for further optimization experiments using the most suitable media.

2.6. Extraction of biotransformation products from fermentation broth

10 ml sample was taken at different time intervals (24, 36, 48, 72, 96 and 120 h), extracted three times with equal volume of ethyl acetate, dried over anhydrous sodium sulphate, and evaporated to dryness under vacuum (55 °C). Then ethyl acetate was added to re-dissolve the residue for analysis. This procedure usually gives an extraction efficiency of >95% [11].

2.7. Analysis of biotransformation products

2.7.1. Qualitative

Biotransformation products in the tested residues were identified via TLC technique. The method adopted can be summarized as follows: samples, dissolved in 100 μl ethyl acetate, were individually spotted (10 μl) by fine capillary on TLC pre-coated silica gel (20 × 20 cm) with fluorescence (Whatmann GF254). The developing solvent system used for TLC was petroleum ether: ethyl acetate (6:4 v/v). Chromatoplates were visualized by short (λ_{254nm}) ultraviolet light, and then sprayed by color reagent, modified Liebermann-Burchard reagent [12] of the following composition; 1 ml sulfuric acid +20 ml acetic anhydride +50 ml chloroform. After spraying, TLC plates were heated at 110 °C for 10–15 min in oven to detect the color of each product according to its authentic color and *Rf* values. The resulting colored spots were viewed in daylight as well as under long (λ_{363nm}) ultraviolet light.

2.7.2. Quantitative

The exact concentration for each product was determined spectrophotometerically using HPTLC (high performance thin layer chromatography). Samples, dissolved in 3 ml ethyl acetate were individually spotted (5 μl) by micro syringe on TLC plates, then the plates were developed in solvent system of petroleum ether: ethyl acetate (6:4) and ultraviolet light absorbance detected at short wave length (λ_{254nm}). The area under the curve of different concentration of the authentic steroid for each product was determined and plotted against concentration and the slope of the straight line was calculated. The unknown concentration of each product was calculated from the following equation:

Concentration = 1/slope × area under the curve × Dilution.

The bioconversion activity percentages (yield of each product) were calculated as follows:

BOL or T or AD% = amount of BOL or T or AD detected/amount of added phytosterol × 100

2.8. Biomass concentration

Twenty milliliters of the broth were extracted with equal amount of chloroform for oil and product removal. After phase separation, the water phase was filtered and twice washed with chloroform and distilled water. The residual cell mass was dried (110 °C) to constant weight.

2.9. Statistical designs

2.9.1. Plackett-Burman design

The Plackett-Burman experimental design is a fractional Factorial design [13]. This design is recommended when more than five factors are under investigation [14]. This design is practical, especially when the investigator is faced with a large number of factors and is unsure which settings are likely to produce optimal or near optimal responses [15]. In this study, the design was used to reflect the relative importance and concentrations of some medium components and fermentation conditions on BOL production. Seven independent variables were screened in nine combinations, organized according to the Plackett Burman design matrix described in the results section. For each variable, a high level (+) and low level (−) was tested. All trials were performed in duplicates and the averages of products percentage were treated as the responses. The main effect of each variable was determined by the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-})/N$$

Where E_{xi} is the variable main effect, M_{i+} and M_{i-} are BOL production in trials where the independent variable (xi) was present in high and in low settings, respectively, and N is the number of trials divided by 2.

2.9.2. Box-Behnken design

In the second phase of medium formulation for optimum BOL production, the Box-Behnken experimental design [16], was applied. In this model, the most significant independent variables, named (X_1), (X_2) and (X_3) were included and each factor was examined at three different levels, low (−), high (+) and central or basal (0). These factors included initial pH of the medium (X_1), concentration of $(NH_4)_2SO_4$ (X_2) and KH_2PO_4 (X_3) were treated as independent variables. Thirteen combinations and their observations (shown in the results section) were fitted to the following second order polynomial mode:

$$Y = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

Where, Y is the dependent variable (BOL production); X_1, X_2 and X_3 are the independent variables; b_o is the regression coefficient at center point; b_1, b_2 and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are second-order interaction coefficients; and b_{11}, b_{22} and b_{33} are quadratic coefficients. The values of the coefficients were calculated and the optimum concentrations were predicted using JMP software. The quality of the fit of the polynomial model equation was expressed by R^2 (regression coefficient).

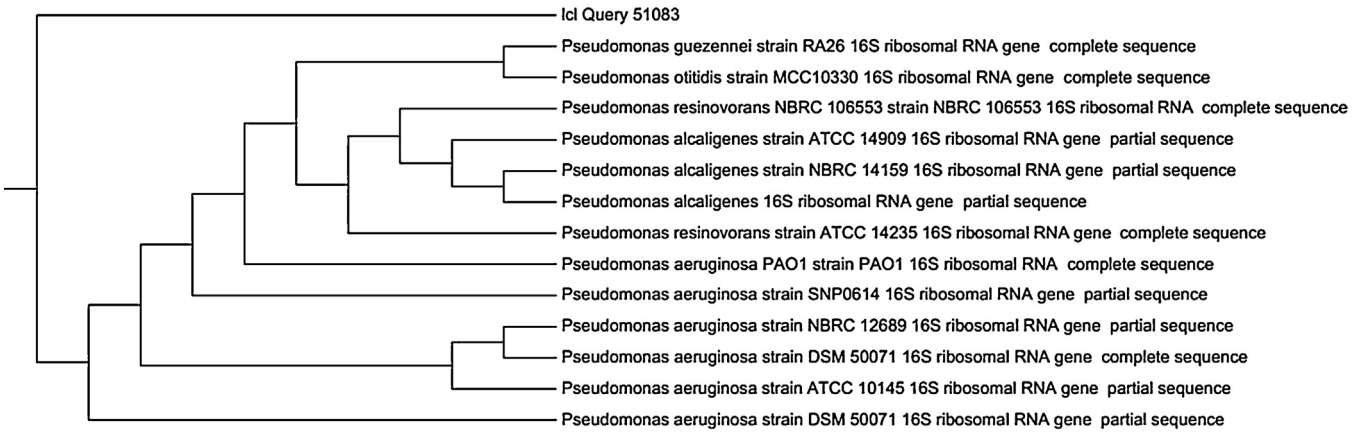

Fig. 1. Phylogenetic tree of isolate B3.

3. Results and discussion

3.1. Identification of microorganisms

Preliminary identification of the isolated strains (Table1) indicated that four of the isolates are *Pseudomonas aeruginosa*. The fifth isolate was identified as *Alcaligenes aquatilis*. This identification is confirmed by API 20NE, VITEK 2 and 16S rRNA gene sequencing. The phylogeny of isolate B3 is indicated in Fig 1.

3.2. Estimation of crude corn oil total sterols

Corn oil total sterol was calculated as 760 mg/100 g oil. This agreed with records by codex standard for naming vegetable oils [17]. Verleyen et al. [18] estimated total sterol content at 779.6–924.3 mg/100 g in crude corn oil, and 685.5–773 mg/100 g in refined corn oil. In addition, crude corn oil total sterol estimated at 700–2210 mg/100 g by CODEX STAN 210-1999 [17]. This relatively high content of the phytosterols is a peculiar feature of corn oil, as compared to other common vegetable oils. Therefore, corn oil represents a cheap, rich raw material that potentially suitable candidate to perform the biotransformation process for the production of steroids using the enzymatic activity of some selected microbial isolates.

3.3. Determination of corn oil phytosterols profile by gas chromatography

GC analysis of corn oil clearly showed its phytosterols mixture profile to be consisted of cholesterol, 2.81%; stigmasterol, 9.03%; campesterol, 16.62% and β-sitosterol 52.8%. In addition, squalene was detected as 6.06% of the corn oil. Squalene is a key intermediate in the biosynthetic pathway of sterols. Majoni and Wang [19] estimated phytosterols of corn oil as stigmasterol, 5.0%; campesterol 7.1%; β-sitosterol 50.9%.

3.4. Screening experiments for biotransformation of corn oil phytosterols

The results of qualitative screening experiments showed that the isolate B1 and B3 have the best biotransformation ability for all the tested products using the three production media. Furthermore, upon quantitative comparison between isolate B1 and B3 for the production of the three products using the NM medium as the production medium, isolate B3 showed the highest transformation yields (Table 2). So, it was selected as the tested organism for further optimization.

As boldenone (BOL) was accumulated as the major product of the biotransformation process, and the rare reports about optimization of its production from phytosterols, it was selected for further optimization. The production of BOL in single step microbial biotransformation from corn oil phytosterols by *P. aeruginosa* was not previously reported. However several authors reported the production of BOL by 1-dehydrogenation of testosterone using *Fusarium lini* [20] or by 17-carbonyl reduction of androst-1, 4-dien-3, 17-dione using *Mucor racemosus* [21]; *Cephalosporium aphidicola* [22]; *Aspergillus brasiliensis* [23]. The proposed biochemical pathway of phytosterol transformation by *P. aeruginosa* in this study involves side chain cleavage of phytosterol to produce AD, which was reduced to T. Both ADD and T were converted to BOL by 17 C=O reduction or 1–2 dehydrogenation respectively.

3.5. Optimization of BOL production by multifactorial designs

The mineral medium [8] was chosen as the production medium for the optimal strategy that involved a two-phase experimental design. The first step was to evaluate the relative importance of various fermentation factors by applying a fractional design. In the second phase, levels of the variables, which have significant influences on BOL production, were investigated.

Table 2
Quantitative comparison between isolate B1 and B3 for biotransformation of corn oil phytosterols. Isolate 3

	Isolate B1								
	AD %	T %	BOL %	Total yield	Biomass g/10 ml	AD %	T %	BOL %	Biomass g/10 ml
24 h	0	0	0	0	0.07	0	0	16.7	0.03
36 h	2.4	1.5	16.26	**20.16**	0.03	7.3	1.4	36.8	0.08
48 h	4.7	3.8	13.7	**22.2**	0.1	10.1	1.6	34.9	0.07
60 h	4.2	7.7	10.3	**22.2**	0.05	9.8	12.4	31.4	0.04
72 h	3.6	5.4	6.4	15.4	0.09	5.3	10.8	26.2	0.05
96 h	2.1	3.4	4.1	9.6	0.06	4.8	7.2	14.7	0.03

Table 3
Factors examined as independent variables affecting BOL production and their levels in the Plackett-Burman experiment and its results.

Trial no	Independent Variables							Response		
	Corn oil %	$(NH_4)_2SO_4$	KH_2PO_4	Na_2HPO_4	Tween 80 %	Initial pH	Time (h)	BOL%	Final pH	Biomass g/10 ml
	X1	X2	X3	X4	X5	X6	X7			
1	−(0.5)	−(1)	−(2)	+(3)	+(0.15)	+(9)	−(36)	17.1	6.55	0.06
2	+(1.5)	−(1)	−(2)	−(1)	−(0.05)	+(9)	+(60)	5.2	5.9	0.06
3	−(0.5)	+(3)	−(2)	−(1)	+(0.15)	−(5)	+(60)	4	3.08	0.02
4	+(1.5)	+(3)	−(2)	+(3)	−(0.05)	−(5)	−(36)	6.2	3.55	0.09
5	−(0.5)	−(1)	+(4)	+(3)	−(0.05)	−(5)	+(60)	3.2	3.18	0.03
6	+(1.5)	−(1)	+(4)	−(1)	+(0.15)	−(5)	−(36)	3.6	3.69	0.11
7	−(0.5)	+(3)	+(4)	−(1)	−(0.05)	+(9)	−(36)	**42.4**	6.49	0.09
8	+(1.5)	+(3)	+(4)	+(3)	+(0.15)	+(9)	+(60)	23.5	6.08	0.17
9	0 (1)	0 (2)	0 (3)	0 (2)	0 (0.1)	0 (7)	0 (48)	38.7	3.82	0.06

3.5.1. Placket-Burman design

The relative importance of various physiological factors involved in the process of corn oil biotransformation was explored using the Placket-Burman design [13] described in the material and methods section. Examined levels of 7 culture variables are presented in Table 3; the design was applied with 9 trials. All experiments were performed in duplicates and the averages of the observations were recorded. The main effects of each variable on AD, T and BOL production, were estimated and expressed graphically (Fig. 2). Data shown in Table 3 illustrated the wide variation in BOL production from 3.2% to 42.4%, thereby reflecting the importance of studying the medium composition for attaining higher productivity. The results showed clearly that BOL production is positively affected by the presence of high levels of $(NH_4)_2SO_4$, KH_2PO_4 and pH adjustment. On the other hand, the incubation time and high concentrations of corn oil, Na_2HPO_4, and Tween 80 showed negative effects on BOL production.

During phytosterols transformation, the pH of the culture broth decreased, Rodina et al. [24] also reported the decrease in pH of the medium during conversion of soybean phytosterols to androstenedione by *Mycobacterium neoaurum*. The results also indicated that at higher substrate concentrations a remarkable decrease in BOL production, this may be due to substrate toxicity. These results agreed with that obtained by Zhang et al. [25] and Abd-Alla [26] as they reported decrease in bioconversion rates at higher levels of the substrate.

Tween 80 is often used in steroid transformation to increase the transport across the cell barrier by increasing bacterial cell permeability [27] and enhance the substrate solubilization in the media surrounding the cell, consequently the concentration gradient [2]. The high concentration of this surfactant showed adverse effect on cell growth and cause decrease in BOL production, as it may be incorporated into membrane lipids and affects microbial surface structure, composition, properties, and functions. Upon studying the effects of Tween 80 on the specific growth rate and biotransformation activities of *Mycobacterium* sp, Tween 80 at 3 g dm^{-3} reduced the specific growth rate by about 40% as compared with the control [28].

The incubation time is critical for steroid biotransformation. Maximum production of BOL in our study was achieved at 36 h (42.4%), on extending the fermentation process to 60 h, a remarkable reduction in BOL% was noticed, The same results were reported by others [7,29]. The results of these experiments indicated that AD and ADD production depended strongly on biotransformation time and substrate concentration [30].

Based on the results obtained from the Placket-Burman experiment, a near optimized medium of the following composition (g/l): corn oil 0.5%; $(NH_4)_2SO_4$, 3; KH_2PO_4, 4; Na_2HPO_4, 1; Tween 80, 0.05%; with an initial pH of 9 and an incubation period of 36 h is used for further optimization of BOL production. In order to determine the accuracy of this experiment, a verification using the experiment was carried out in a triplicate. The predicted near optimum levels of independent variables were examined against the basal condition settings, BOL yield increased from 36.8% to 42.4%.

3.5.2. Box-Behnken design

In order to approach the optimum response of BOL production, the effective independent variables, including the initial pH (X_1), and the concentrations of $(NH_4)_2SO_4$ (X_2) and KH_2PO_4 (X_3), were further investigated each at three levels according to the Box and Behnken design [16]. However, other variables with less significant

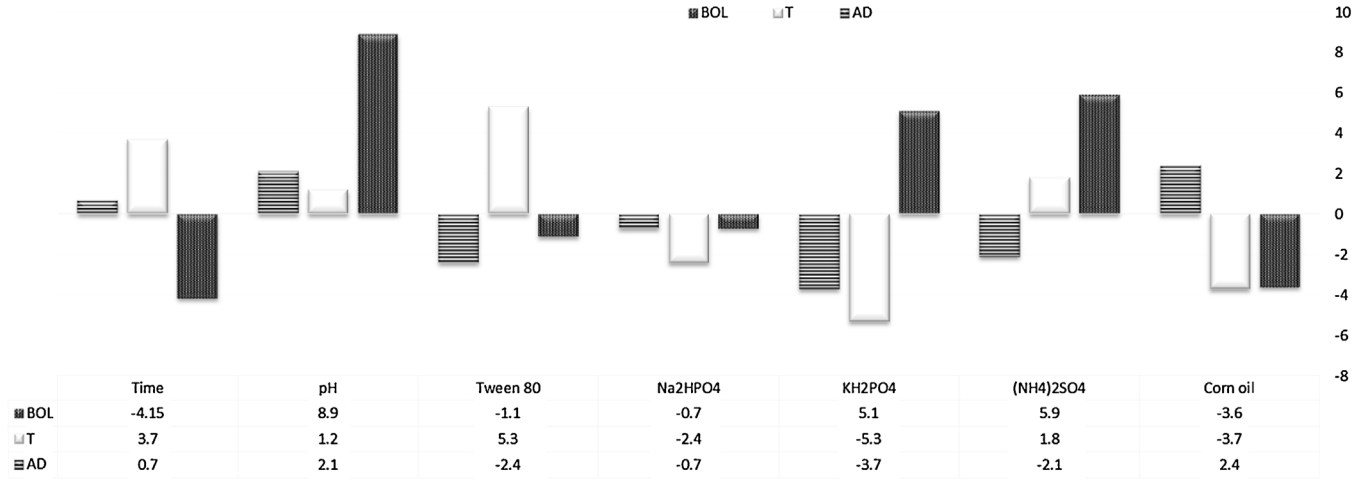

Fig. 2. Main effects of independent variables on BOL production according to the results of the Plackett-Burman experiment.

Table 4
Box–Behnken factorial design for three independent variables.

Trial	Independent Variables			Response		
	pH X1	$(NH4)_2SO_4$ X2	KH_2PO_4 X3	BOL %	Final pH	Dry Weight g/10 ml
1	8 (−)	2 (−)	4 (0)	**53.6**	5.93	0.041
2	10 (+)	2 (−)	4 (0)	1.8	7.61	0.096
3	8 (−)	4 (+)	4 (0)	30	6.14	0.063
4	10 (+)	4 (+)	4 (0)	2.6	9.85	0.08
5	8 (−)	3 (0)	3 (−)	26.3	5.69	0.091
6	10 (+)	3 (0)	3 (−)	5.3	9.66	0.059
7	8 (−)	3 (0)	5 (+)	20.2	6.28	0.093
8	10 (+)	3 (0)	5 (+)	0	9.7	0.059
9	9 (0)	2 (−)	3 (−)	22.4	5.85	0.111
10	9 (0)	4 (+)	3 (−)	43.1	5.95	0.101
11	9 (0)	2 (−)	5 (+)	26.9	6.41	0.11
12	9 (0)	4 (+)	5 (+)	26.3	6.44	0.129
13	9 (0)	3 (0)	4 (0)	51	6.16	0.105

effects that had a negative effect on BOL production were not included in Box-Behnken optimization experiment, but instead were used in all trials at their (−) level. According to these results, the experiments were designed to obtain a quadratic model consisting of 12 trials and 1 center point. Table 4 represents the design matrix of the coded variables together with the response of BOL production, final pH and growth.

As shown in Table 4 the highest BOL yield 53.6% obtained at pH 8 that was the optimum pH. The results also indicated that upon increasing the medium pH to 10 a sharp decrease in BOL production occurred. Optimum pH 8 was also reported by Goswami et al. [31] for production of AD (D) from 17-ketosteroids by *Arthrobacter*. In addition, optimal pH between 6 and 8 for steroid biotransformation had referred by several reports as Sallam et al. [7] and Zhang et al. [25]; they reported optimal pH at 7. Abd-Elsalam et al. [30] reported optimum pH 6.5 for production of androstenedione (AD) and androstadienedione (ADD) from sunflower oil sterols by *Fusarium solani*. Adequacy and fitness of the model were evaluated by ANOVA analysis (Table 5). The regression equation coefficients were calculated, and the data were fitted to a second-order polynomial equation. The regression equation obtained was as follows:

$$YBOL = 51 - 15.05\,X_1 - 0.3375\,X_2 - 2.9625\,X_3 + 6.1\,X_1X_2 + 0.2\,X_1X_3 - 5.325\,X_2X_3 - 22.8625\,{X_1}^2 - 6.1375\,{X_2}^2 - 15.1875\,{X_3}^2$$

Where YBOL is the predicted response of BOL yield and X_1, X_2 and X_3 are the coded independent variables for initial pH, the concentrations of $(NH_4)_2SO_4$ and KH_2PO_4 respectively. The F value and P values were used to check the significance of each coefficient, which also indicated the interaction strength between independent variables. The larger the magnitude of the *F* value and smaller the *P* value, the more significant is the corresponding coefficient [32]. ANOVA analysis showed a significant *F* value (22.0627) for the effect of pH on BOL production, which implied the model to be significant. The model has a value of Probability P (0.0054) less than 0.05 that considered highly significant. The determination of coefficient (R^2) was calculated as 0.921023 for BOL production (a value of $R^2 > 0.75$ indicated the aptness of the model) which indicates the statistical model can explain 92.1% of variability in the response. The goodness of the model can be checked by the determination of coefficient (R^2) and correlation coefficient (R). The closer the value of R to 1, the better the correlation between the measured and the predicted values. It can be seen from Table 5 that the linear term regression coefficients of X_1 are highly significant at the 5% level, these results indicated that the initial pH of the medium is correlated in direct relationship with the production of BOL. An overall 45.6% increase in BOL yield has being achieved after application of RSM. This reflects the necessity and value of the optimization process. The effect of X_2 and X_3 as well as their interaction effects were not significant ($P > 0.05$).

Three dimensional response surface plots (Fig. 3) and Pareto Plot (Fig. 4) were employed to determine the interactions of the three factors and showed the optimum levels of each and their interactions. The optimal levels of the three factors were estimated using SAS JMP 8 NULL program tools and found to be as follows: pH 8; $(NH_4)_2SO_4$; 2 g/l and KH_2PO_4; 4 g/l. Our result showed graphically, the relationship and the interaction between the independent variables (initial pH, the concentrations of $(NH_4)_2SO_4$

Table 5
Results of ANOVA analysis for optimization of BOL production by the Box–Behnken experiment.

Term	Coefficient estimate	*df*	SE	Sum of squares	*t*-value	*F*-value	*P*-value
Corrected Model		9		4788.9808		6.4788	0.0267
Intercept	51	1	5.232288		9.75		0.0002
X_1 pH	−15.05	1	3.204109	1812.0200	−4.7	22.0627	0.0054[a]
X_2 $(NH_4)_2SO_4$	−0.3375	1	3.204109	0.9113	−0.11	0.0111	0.9202
$X_3KH_2PO_4$	−2.9625	1	3.204109	70.2113	−0.92	0.8549	0.3976
X_1[a] X_2pH*$(NH_4)_2SO_4$	6.1	1	4.531294	148.8400	1.35	1.8122	0.2360
X_1[a] X_3pH[a] KH_2PO_4	0.2	1	4.531294	0.1600	0.04	0.0019	0.9665
X_2[a] X_3 $(NH_4)_2SO_4$[a] KH_2PO_4	−5.325	1	4.531294	113.4225	−1.18	1.3810	0.2928
${X_1}^2$ pH[a] pH	−22.8625	1	4.71632	1700.0298	−4.85	20.6991	0.0047[a]
${X_2}^2$ $(NH_4)_2SO_4$[a] $(NH_4)_2SO_4$	−6.1375	1	4.71632	91.7178	−1.30	1.1167	0.2499
${X_3}^2$ KH_2PO_4[a] KH_2PO_4	−15.188	1	4.71632	851.6683	−3.22	10.3697	0.0635

R^2 = R Squared = 0.921023 (Adjusted R Squared = 0.778864).
[a] Significant at 5% level.

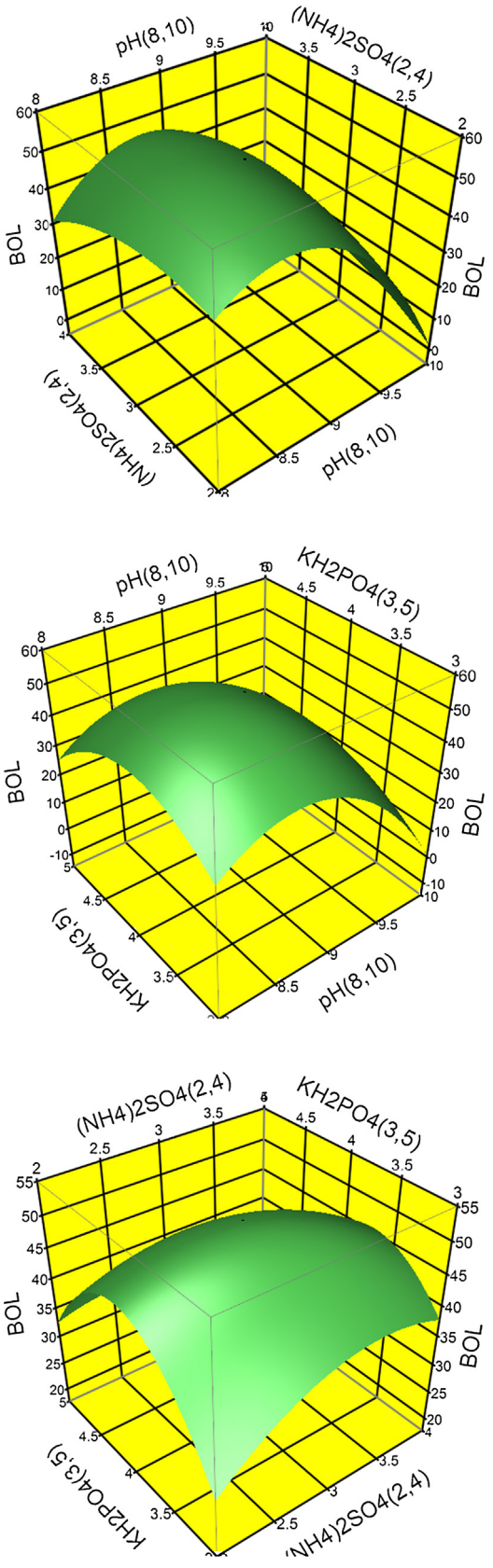

Fig. 3. Three-dimensional surface plots showing the relationships between significant tested factors on BOL yield.

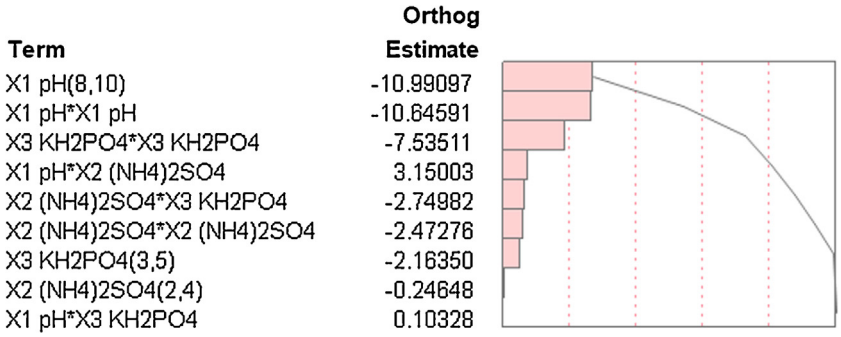

Fig. 4. Pareto Plot of Transformed Estimates.

and KH_2PO_4) and response (BOL production). These factors are the most important factors and needs fine regulation for enhancing BOL production.

4. Conclusions

Conclusions In this study, A new potent *Pseudomonas aeruginosa* isolate capable of biotransformation of corn oil phytosterol (PS) to 4-androstene-3, 17-dione (AD), testosterone (T) and boldenone (BOL) was identified by phenotypic analysis and 16S rRNA gene sequencing. The response surface methodology was a potent and efficient method to optimize the parameters affecting the PS biotransformation process. To our knowledge, the microbial production of (BOL) from corn oil phytosterols in single step biotransformation process by *P.aeruginosa* was not previously reported. By analyzing the statistical model of three-dimensional surface plot, BOL production increased from 36.8% to 42.4% after the first step of optimization, and the overall biotransformation increased to 51.9%. After applying the second step of the sequential statistical strategy BOL production increased to 53.6%, and the overall biotransformation increased to 91.9% using the statistically optimized medium.

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