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Research Article

DETERMINATION OF REDUCTION IN CITRININ PRODUCTION BY RESPONSE SURFACE METHODOLOGY USINGAQUEOUS LEAF EXTRACT OF *NEPETABRACTEATA*

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Abstract: Citrinin (mycotoxin), a nephrotoxic agent produced by many fungal strains belonging to the genera *Penicillium*, *Aspergillus*, and *Monascus*. Generally found in stored grains and after their harvest. The objective of the present investigation was to study and optimize the conditions for maximum inhibition of citrinin and fungal biomass by using aqueous leaf extract of *Nepetabracteata*under laboratory conditions by *Penicilliumcitrinum*.Optimization of culture conditions was carried out using Box-Behnken method of response surface methodology. Extent of inhibition of citrinin was carried out using HPLC and reduction in fungal biomass was carried out using dry cell weight after comparing with controls. Optimized culture conditions as per the point prediction tool were found to be 10.32mg/L for concentration of extract*Nbracteata*, ten days of incubation period and temperature of 24.5 °C resulted in maximum inhibition of Citrinin. These optimized values of tested parameters were and compared with control citrinin production (239.85 mg/L) and dry cell weight production (386.02mgAn average of 88.38±2.09% inhibition of citrinin and 79.89±0.94% of dry cell weight was obtained in an optimized medium at 10 d of fermentation with 95.26 % and 92.89% validity, respectively.

Key**words:** *Penicilliumcitrinum*, Citrinin, fungal biomass, *Nepetabracteata*, Box-Behnken design

1. Introduction

Mycotoxins are secondary metabolites produced by microfungiand are capable of causing disease and death in humans and other animals. Because of their pharmacological activity.Citrinin [518-75-2], 4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-crboxylic acid[1],which was first isolated by HetheringtonandRaistrick from a culture of *Penicilliumcitrinum*Thom[2], Citrinin is the one of the well-known mycotoxins, which is possibly spread all over the world. While citrinin is one of the well-characterized mycotoxins, information on its mechanism of toxic action is limited. Clinically, citrinin was shown to cause renal disease in poultry, pigs, dogs and rats[3,4].Several other fungal specieswithin the three genera*Penicillium*[5], *Aspergillus*[6]and *Monascus*[7,8]were also able to produce citrinin[9,10].Due to its antibacterial potential, citrinin was used as an antibiotic (Wong and Koehler 1981).Citrininncontaminates maize[11], wheat, rye,barley, oats[12], and rice[13].

The plant *N bracteata* of family Laminaceaeis an aromatic perennialherbaceous plant has been the subject of interest because of its medicinal importance.It is a brightly colored shrub and 30-100 cm in height.Nepeta is a large group of hardy herbaceous plants found in different parts of Asian region like Pakistan, India, China, and Bangladesh. Leaves are ovate-obtuse[14].Theplant yields bunchof pink blue and more rarely white fragrantflowers during summer[15,16].A many number of research work concerning pharmacological, phytochemical, toxicological studies has been reported. Syrup prepared from theleaves, flowers and a seed is reported to beuseful in cough and fever, Antimicrobial activity of *Nbractaeta*has also been reported[17]. Itis also reported to be used in, boils and abscesses,cystitis, gastritis, fever, rheumatism,cold[17] cough, asthma,earache, insect bites, flatulence[17, 18]. The major classes' ofchemical constituents present in this plantare carbohydrate, flavonols, phenolics, saponins[19].But more work needs to be done regarding its medicinal importance, taxonomic and ecological aspects. As the plant of *N bracteata*has immense potential as an antimicrobial due to the rich source of phytochemicals it possesses, therefore, such studies on biological activities particularly anti-microbial are recommended in various parts of the Asian subcontinent where it grows.

Response surface methodology (RSM) is a statistical technique used for the development and optimization of complex processes[20, 22]. It was selected and used to find the optimum conditions for maximum inhibition of citrinin and dry cell weight produced byfungus *P citrinum*.The technique has several advantages over conventional experimental or optimization methods in which one variable at a time is used. RSM provides a large amount of information and is more economical approachbecause; a small number of experiments are performed for monitoring the interaction of the independent variables on the response. In conventional optimization, the increase in the number of experiments necessary to carry out the research, leads to an increase in time and expenses as well as an increase in the utilization ofreagents and materials for experiments. The equation of the model easily clarifies the effects for binary combinations of the independent variables. Box–Behnken design is advantageous because it does not contain any points at the extremes of the cubic region created by the two-level factorial level combinations

that are prohibitively expensive or impossible to test because of physical constraints on experimentation[23].

Medicinal plants represent an important health and economic component ofbiodiversity. In the present investigation the Box Behnken design was selected and used to optimize the aqueous extracts of tested medicinal plant on growth of *Penicilliumcitrinum*on citrinin production and dry cell weight.

2. Experimental Methods

2.1 Microorganisms and culture conditions:

Citrinin-producing microorganisms namely*Penicilliumcitrinum*was obtained from the culture collection of the Microbiology Laboratory of King Khalid University Hospital, King Saud University, Riyadh; KSA. The fungal culture was maintained on slants of potatodextrose agar medium at 4°C. The spore was suspended by growing the fungi on Petri dishes for 7 days at 25°C with potato dextrose agar (PDA) containing 50 mg/L of streptomycin. Later Spores were harvested by adding 10 ml of sterilized distilled water on each plate. The spore suspension hence obtained was filtered using cheesecloth, and spores were counted using a haemocytometer and brought to a final concentration of 10^5 conidia/ml.

2.2 Collection and preparation of aqueous plant extracts

Literature survey was done and taxonomic studies of the herbarium specimens of the medicinal plants available at the National Herbarium of Saudi Arabia (RIYADH), and the herbarium of King Saud University (Pharmacy), was done by using long arm stereomicroscope. Leaves of the *Nepetabracteata*were collected and washed under tap water. Then the leaves were dried at 60°C in hot air oven for 5 days and ground to make a powder and passed through 20 mesh sieve. Ten grams of powdered leaves were made soluble with 100 ml distilled water at 200 rpm for 5 h at room temperature[24]. The remaining insoluble material was filtered by Whattman No.1 filter paper and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and again passed through 0.45 µm filter (Millipore) and stored at -20°C for further use.

2.3 Seed culture and fermentation

The culture of *P citrinum* was grown onpotato glycerol agar (PGA) slants and spore suspension(4×10^6 spores per mL) was made inglycerol water solution (15 g l^{-1}). The seed culturemedia used in this study were glucose (20g l^{-1}), glycerol (30 g l^{-1}), peptone (8 g l^{-1}),$NaNO_3$ (2 g l^{-1}) and $MgSO_4$ (1 g l^{-1}) were dissolvedin water-soluble extract of soybean meal. All fermentation experiments were carried outin 250 ml Erlenmeyer flasks containing 50 ml ofproduction media as per experimental design[25].

Different concentrations of aqueous plant extract were added to liquid broth. 10 µl amount from suspension (containing 10^5 spore/ml of *P citrinum*) was inoculated in each flask and kept in rotary shaker at 200 rpm. The control contained production medium and 10 µl of *P citrinum*suspension. The fungal mycelium was harvested by filtration by muslin cheese cloth to separate from liquid culture. The filtered biomass was then dried at 60°C for 24h and the dry weight of mycelium was determined. Filtrates were stored in 4°C for carrying out citrinin extraction. All experiments consisted of three replicates, and the averages were determined.

2.4 Extraction and quantification of citrinin

The filtrates obtained from above experiment were used for extraction and estimation of citrinin. Briefly, the citrinin was extracted thrice with chloroform (1:1 *v/v*). All the three extractions were pooled and concentrated in vacuum at 40°C using a rotary evaporator. All the concentrates were then diluted in mobile phase (2ml) and citrinin was estimated by HPLC.All the samples were filtered through a 0.22µm disposable syringe filter (Micro Filtration Systems®) prior to injection into the chromatograph. Aliquots (30 µl) were injected on HPLC column and analysis were carried out using a Shimadzu® Liquid Chromatograph, equipped with an LC-20AD pump, a Rheodine® injector, an SPD-20A UV detector, a CBM-20 A-Communications Bus Module, and a LC Solutions Workstation system. A reverse-phase Atlantis® dC 18 column (150 × 3.9 mm, 5 µm) was used, at room temperature. The mobile phase used was acetonitrile-water (75:25 *v/v*) and formic acid (2%) with a flow rate of 1.5 mL/min for an isocratic run of 10 min. Absorbance of samples and standard was detected at 360 nm[26]. Retention times and peak areas were calculated by LC Solutions software. Evaluation of sample retention times with that of the standard identified the presence of citrinin in the samples. The relationships between peak area and the amount injected were linear over the ranges 2.5-50 µg.

3. Optimization of experimental conditions

3.1 Box–Behnken experimental design

A response surface statistical experimental design was used to optimize the concentration of extract, incubation days and temperature. This design was based on a 3^3 factorial design, three replicates of the central run, leading to 15 sets of experiments, enabling each experimental response to be optimized. The responses were investigated using a Box–Behnken statistical experimental design. The optimization process involves evaluating the response of the statisticallydesigned combinations, estimating the coefficients by fitting the experimental data to the response function, predicting the response of the fitted model, and checking the adequacy of the model. All experiments were performed in standard order to minimize the effects of uncontrolled factorsthat may introduce a bias in the response. Before starting an optimization procedure, it is important to identifythe crucial factors affecting the quality of the derived outcomes. The levels of the three factors evaluated in this design are listed in (Table 1). A three factor, three-Level Box–Behnken design was used for the optimization procedure, using the software Design Expert V 8.0.7.1. All other factors, for example volume of spore suspension, pH of the broth etcwere maintained constant. The quality of the fitted model was expressed by the coefficient of determination R^2, and its statistical significance was checked by an *F*-test (analysis of variance) at the 5% significance level. The optimum processing conditions were obtained by using graphical and numerical analysis based on the criteria of the desirability function and the response surface.The experiment was finally repeated under the optimum values as per the point prediction tool of

response surface methodology for concentration of extract, incubation period and temperature which should result in maximum inhibition of citrinin and dry cell weight production. These optimized values of tested parameters were validated (n=6) and compared with control for citrinin and dry cell weight inhibition.

4. Results and Discussion

4.1 Design of the proposed assay and strategy for its development

The main aim of the optimization process was tomaximize the response value in the extracts i.e., to optimize the most appropriate conditions for maximum inhibition of citrinin production and fungal biomass by using medicinal plant aqueous extract of*Nbracteata*. The suitabilityof the model equation for predicting the optimumresponse value was tested using the selectedoptimal conditions. The results for the threeanalyzedparameters and the maximum predicted yield andexperimental yield of polyphenols are given in Table 2.The levels of inhibition of citrinin was assessed by HPLC in samples treated with extract of *N bracteata* and the percentage of inhibition compared to the control (Fig 1), similarly the inhibition in biomass was studied by dry cell weight and compared with control.

4.2 Optimization of fermentation medium

The key parameters most influencing on the inhibition of citrinin and fungal biomass, *viz*concentration of plant extract, incubation days and temperature were studied. The results of experimental runs are summarized in (Table 2). Data collected from experimental runs were analyzed by using the software Design Expert V 8.0.7.1and fitted to nonlinear quadratic models for citrinininhibitionand fungal biomass analysis.The model was validated by analysis of variance (ANOVA). The statistical analysis showed that the model represents the phenomenon quite well and the variation of the response was correctly related to the variation of the factors (Table 3).In general, exploration and optimization of a fitted response surface may produce poor or misleading results, unless the model exhibits a good fit, which makes checking of the model adequacy essential[27]. The F-ratio in this table isthe ratio of the mean square error to the pure error obtained from the replicates at the design centre. The significance of the F-value depends on the number of degrees of freedom (DF) in the model and is shown in the P-value column (95% confidence level). Thus, the effects lower than 0.05 are significant[28] (Morelli, 2012). The P-value is used as a tool to check the significance of each coefficient and the interaction strength between variables[29]. The higher the significance, the better the degree of correlation between the observed and predicted values[30]. Total extract concentration, incubation days and temperature content were significantly affected.

An experimentaldesign of 15 runs containing 3centralpoints was made according to Box-Behnken of responsesurface methodology to optimizethesemedium parameters. The individual and interactiveeffects of these parameters werestudied during fermentation. The response wasmeasured in terms of actual factors of inhibition of citrininanddry cell weight. Data collected from experimental runs wereanalyzed by using the softwareand fitted tononlinear quadratic models for citrinin and dry cell weight inhibition.The regression analysis was carried out to fit mathematical models to the experimental data aiming at an optimal region for the responses studied. Predicted response Y for the yield of each response could be expressed by the following polynomial quadratic equation in terms of actual values: The significance of each coefficient is listed in Table 4. The fitted model equation is:

Citrinin inhibition (mg/L) = - 85.90816+ 6.33673A+ 53.69494B - 23.30952C-0.633AB - 0.22857AC + 0.0375BC +0.26701A^2 - 1.16276B^2 + 0.50333C^2. Biomass (mg) =+481.7576531+14.2244A +46.2120B -63.2607C - 0.78571AB -0.65714AC +0.09375BC +0.35459A^2- 0.865234B^2+ 1.385 C^2

This multiple nonlinear model resulted inthree response surface graphs each for citrininand dry cell weight. Point predictiontool of the software was used to calculatemaximuminhibition of citrinin and dry cell weight. Finally the optimumvalues as per the point prediction tool were found to be 11.27 mgl^{-1} for concentration of extract, nine and half days of incubation period and temperature 25.5°C which resulted in maximum inhibition or minimum production of citrinin(27.62mgl^{-1}) and dry cell weight production (76.82 mg).Theseoptimized values of tested parameters werevalidated under similar conditions (n=6) as discussed in material and methods and compared with control citrinin production (239.85 mgl^{-1}) and dry cell weight production (382.02mg). An averageof88.38±2.09% inhibition of citrininand79.89±0.94% of dry cell weight was obtained in anoptimized medium at 10d of fermentation with95.26% and 92.89% validity, respectively.

The relationship between independent and dependent variables is illustrated in three-dimensional representation of the response surfaces and one factor plots generated by the models for citrinin and biomass (Fig. 2).*Figure 2A and B*shows the effect of extract concentration and incubation days on citrinin and fungal dry cell weight at a constant temperature 25°C. It is apparent from the figure that citrinin and fungal dry cell weight inhibition increased with increasing extract concentration and number of incubation days. *Figure 2C and D*shows the effect of temperature and concentration of extract on inhibition of citrinin and fungal dry cell weight at fixed incubation period of 16 days.*Figure 2E and F*shows the effect of temperature and incubation days of citrinin and fungal dry cell weight at fixed concentration plant extract(9mgl^{-1}).Figure 3AI, AII;BI, BIIandCI, CII shows the effect of individual factors for extract concentration, incubation days and fermentation temperature of both citrinin and fungal dry cell weightis evident from the figures that both these variables have direct effect citrinin concentration and fungal biomass.The results of analysis confirmed thatthe response model was adequate for reflecting theexpected optimization, and the model was satisfactoryand accurate.

5. Conclusion

The present research study was designed to identify the antifungal activity of natural products for inhibition of citrinin in food grains. The antifungal potential of the *N*

*bracteata*was studied for the first timefor preservation of food grains from production of citrinin, a known mycotoxin.Citrinin is a proven hepatonephrotoxic agent and hence need to be inhibited. The effect of individual variables on inhibition of citrinin and fungal dry cell weight was also studied using Box-Benkhen response surface methodology. Optimum conditions for maximum inhibition of mycotoxincitrinin were also developed. The results obtained using response surface predictions were in good agreement with the experimental results. Therefore, Box–Behnken statistical design used in determining the optimum experimental conditions such as extract concentration, incubation days and temperature was reliable and effective. Hence extracts of *Nepetabracteata* can be used as a preservative in food grains for prevention of any toxic fungal secondary metabolite because of its potential antimicrobial activity.

Table 1: Levels of tested parameter for Box-Behnken design

Independent factors	Unit	Symbol	Levels	
			Low	High
Extract Concentration	mg/L	A	2	16
Incubation Time	Days	B	8	24
Temperature	°C	C	20	30

Table 2: The Box–Behnken design matrix of three variables

Runs	Factors			Citrinin (mg/L)		Fungal biomass (mg)	
	Extract Conc. (mg/L)	Incubation Days	Temperature (ºC)	Actual	Predicted	Actual	Predicted
1	2	8	25	4	0.75	85	83.37
2	16	8	25	1	5.75	56	53.87
3	2	24	25	264	259.25	390	392.12
4	16	24	25	119	122.25	185	186.62
5	2	16	20	203	207	301	296.87
6	16	16	20	161	157	229	225.37
7	2	16	30	223	227	355	358.62
8	16	16	30	149	145	191	195.12
9	9	8	20	3	2.25	76	81.75
10	9	24	20	186	186.75	293	295
11	9	8	30	4	3.25	92	90
12	9	24	30	193	193.75	324	318.2
13	9	16	25	156	158.34	211	217
14	9	16	25	158	158.33	221	217
15	9	16	25	161	158.33	219	217

Table 3. Analysis of variance of calculated model for citrinin and biomass.

	Citrinin	Biomass
Model (Mean Square)	11910.91	17083.51
Residual	145.17	204.75
R^2	0.998	0.999
F	410.25	417.18
p-value	< 0.0001	<0.0001
± SD	5.39	6.39
RSD	4.07	2.97
*Adequate Precision	58.76	64.74

*Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. This model can be used to navigate the design space.

Table 4: Regression coefficients and their significance in the quadratic model

Terms	Citrinin		Biomass	
	Coefficient Estimate	***P-value***	**Coefficient Estimate**	***P-value***
Intercept	158.33	< 0.0001	217	< 0.0001
A- Extract Conc.	33	< 0.0001	-58.75	< 0.0001
B- Incubation Time	93.75	< 0.0001	110.375	< 0.0001
C- Temperature	2	0.3419	7.875	0.0176
AB	35.5	< 0.0001	44	< 0.0001
AC	8	0.0312	23	0.0008
BC	1.5	0.6017	3.75	0.2940
A^2	13.08	0.0055	17.375	0.0034
B^2	74.42	< 0.0001	55.375	< 0.0001
C^2	12.58	0.0065	34.625	0.0001

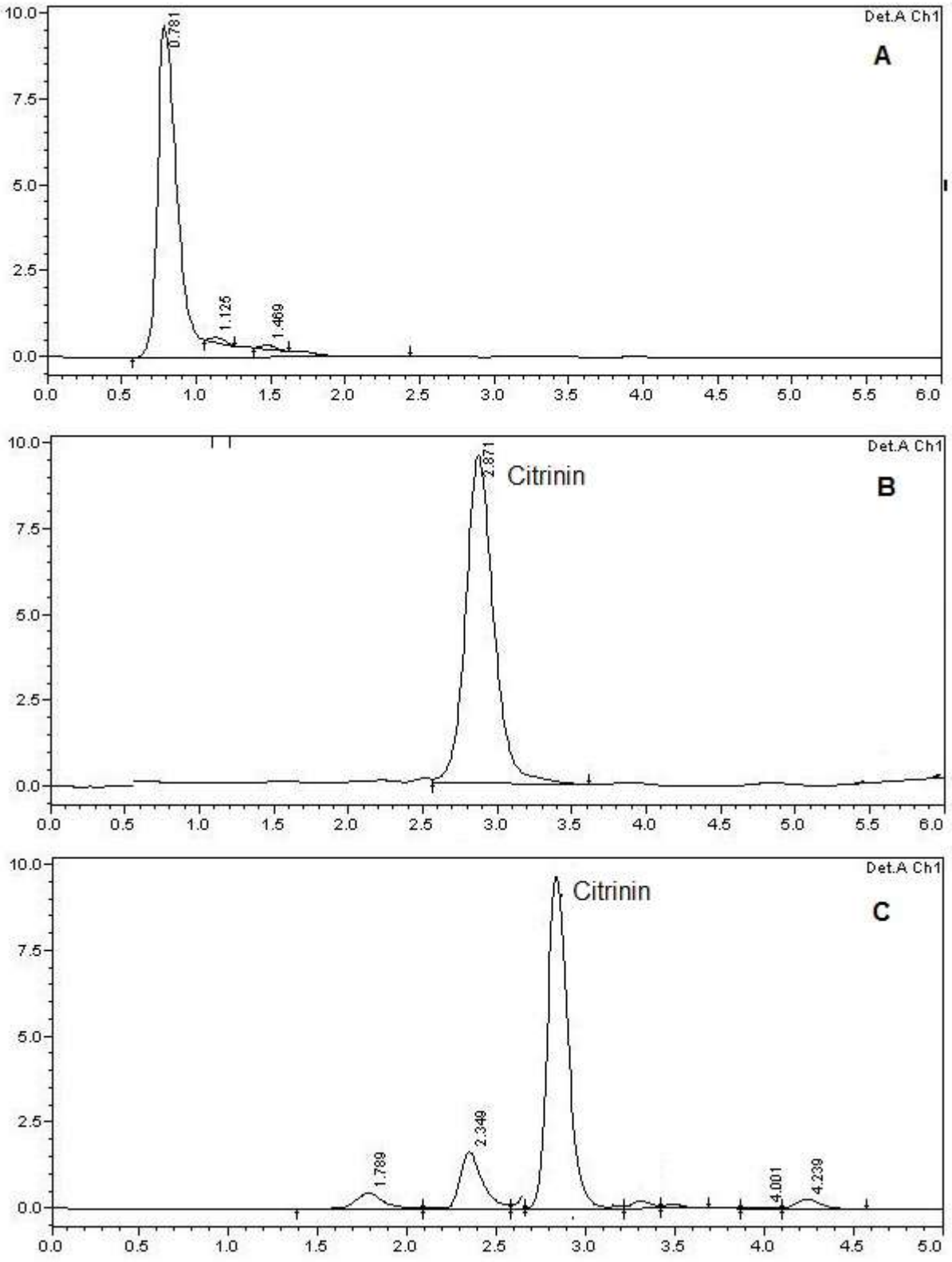

Figure 1 HPLC chromatograms of citrinin a) blank sample b) Standard citrinin (RT=2.871) c) fermented sample (RT=2.879).

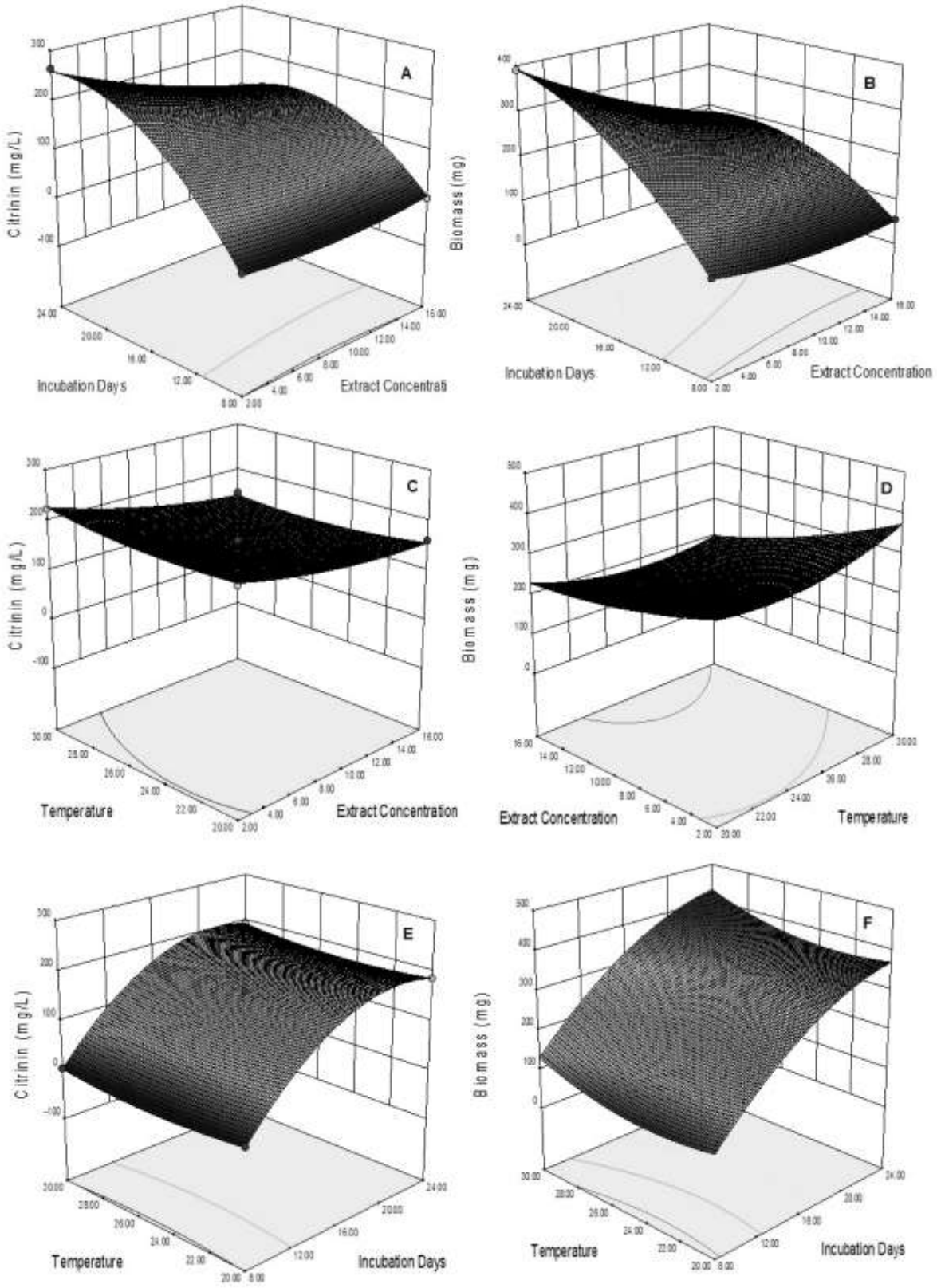

Figure 2: Three dimensional response-surface graphs representing the effect of extract concentration, incubation time and temperature on the responses: (AI and AII) Relative effect of extract concentration and incubation time on citrinin and biomass production, respectively at constant temperature. (BI and BII) Relative effect of temperature and extract concentration on citrinin and biomass production, respectively at constant concentration of extract.

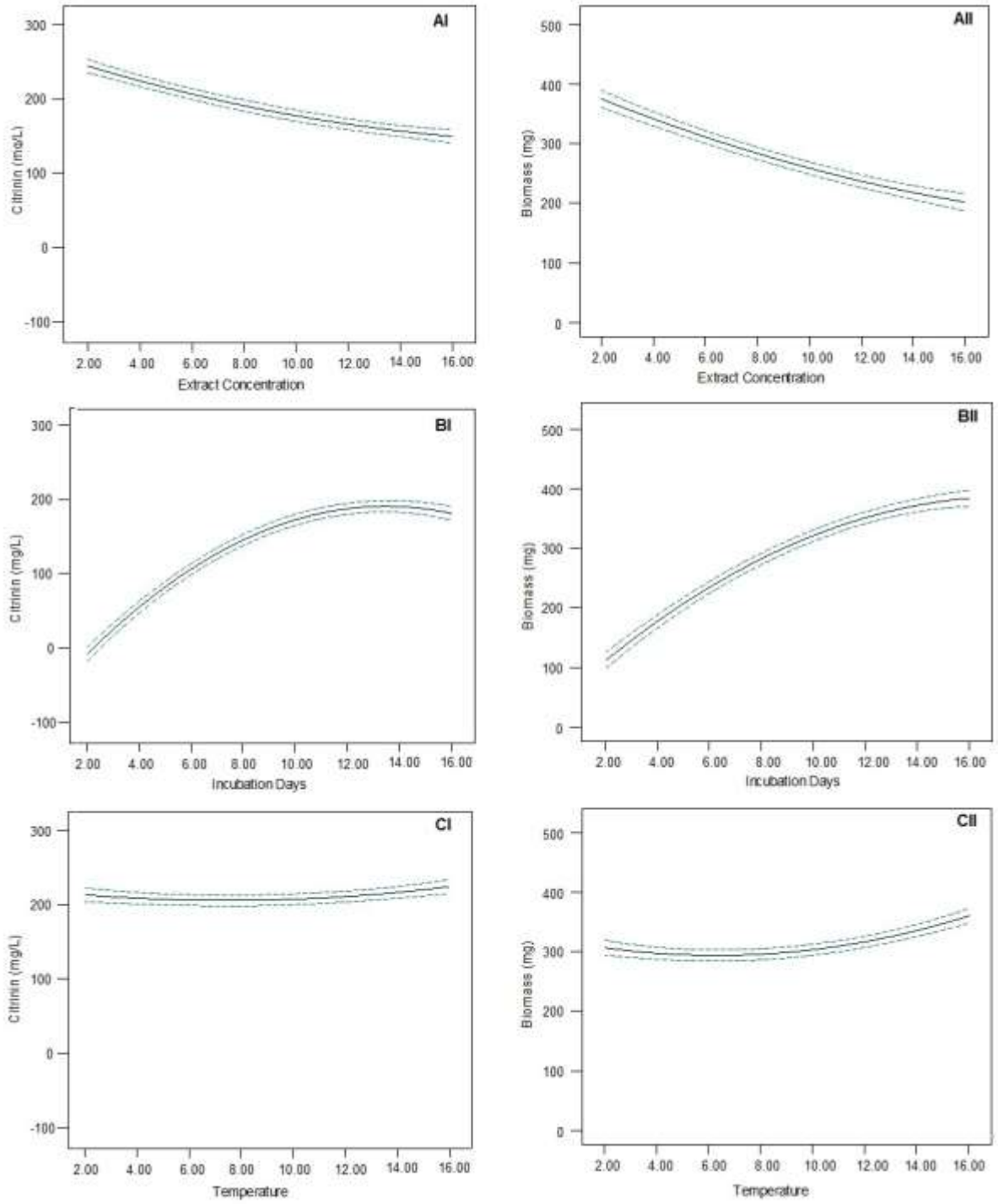

Figure 3: Effect of individual factors extract concentration (AI and AII), incubation time (BI and BII) and temperature (CI and CII)oncitrinin and biomass production

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