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Optimization of the Elicitation Process on *Chrysanthemum indicum* Cell Suspension Culture Producing Xanthine Oxidase Inhibitor

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Abstract: This research reports the study that aims to enhance the activity of xanthine oxidase inhibitor, a secondary metabolite, found in *Chrysanthemum indicum* and to identify the most suitable elicitors with the optimize elicitation time and concentration. Many plant species are well known source of secondary metabolite with a variety of biological activities. Production of biologically active compounds using plant cell suspension culture approach, normally gives low result. Optimization studies to enhance xanthine oxidase inhibitor activity using different elicitor concentrations for three different types of elicitor (chitosan, yeast extract and *Aspergilus niger*) were investigated. A factorial design was used from MINITAB software for optimization of the process. Analysis of results proved that chitosan is the best elicitor to increase activity of xanthine oxidase inhibitor in *C. indicum* to 1.457 fold higher than the unelicited. The highest xanthine oxidase activity was obtained by chitosan elicitation at concentration of 0.16 g L^{-1} and it was elicited at 1.25 day cultivation or at 30 h culture with percentage inhibition of 61.818%.

Key words: Chitosan, yeast extract, *Aspergillus niger*, surface response methodology, anti-gout

INTRODUCTION

Various parts of Chrysanthemum are used as anti-inflammatory and blood purifier medicines. The inflorescence or bud of *Chrysanthemum indicum* has long been used as a Chinese traditional medicine, particularly for the treatment of inflammation, hypertension and respiratory diseases. Earlier studies have report that *Chrysanthemum indicum* possesses anti bacterial, anti virus, anti oxidant, anti-inflammatory, immunomodulatory (Wang *et al*., 2000) and anti-gout properties at high xanthine oxidase inhibition activity (Nguyen *et al*., 2004). However, *C. indicum* needs a suitable growing area such as highland for them to give flowers consistently throughout the year, thus, most of the lowlands in Malaysia are not suitable for this activity.

In recent years, various plant cell-culture systems have been exploited for the enhancement of high value metabolites. However, looking at the low production of this secondary metabolite and scarcity of the source material, it is suggested that it is not so viable. In view of this, studies on the enhancement of xanthine oxidase inhibitor using plant-cell and tissue-culture together with elicitor have been carried out. Numerous approaches have been developed to enhance the productivity of plant-cell and tissue-culture such as medium optimization, cell line selection, cell immobilization, precursor addition, elicitation, genetic transformation, organ or hairy root cultures, metabolic engineering and integrated bioreactor engineering. Elicitation was most successful in cell-culture of various plant species in order to boost the production of secondary metabolites in plants (Abdullah *et al*., 2005).

Elicitation is a method adopted for the purpose of enhancing the secondary metabolite production. It is defined as the induction of secondary metabolite production by molecules or elicitor treatment (Singh, 1999). The recent development of elicitation has opened a new avenue in the area of production of secondary metabolites (DiCosmo and Misawa, 1985). The elicitors prepared from *Rhizoctonia solani,* improved the solavetivone production in *Hyoscyamus muticus* (Ramakrishna *et al*., 1993) and *Asperigillus flavus* mycelial extract elicited anthocyanin content in *Daucus carota* cell-culture (Rajendran *et al*., 1994). It has also been reported that increased levels of kalopanaxsaponin in leaves of *Nigella sativa* by methyl jasmonate (Scholz *et al*., 2009) and chitosan induced anthraquinones production in *Rubia tinctorum* suspension cultures (Perassolo *et al*., 2008). Use of elicitors, which are not specific to the species or an inappropriate production

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medium, can cause ineffective elicitation. Funk and Brodelius (1990) failed to induce the phenylpropanoid pathway in cell-suspensions of *Vanilla planifolia* by using yeast elicitor.

Thus, successful application of elicitation is a challenging task and requires an intensive and prolonged trial and error procedure. Despite the importance of elicitation for enhancing accumulation of secondary products and for a better understanding of their biosynthesis and regulation, such studies have never been carried out on *C. indicum* cultures. Therefore, in order to induce more xanthine oxidase inhibitor activity, different kinds of inducers (elicitor) have been applied in a stipulated amount and at the suitable time.

MATERIALS AND METHODS

This study was carried out in Faculty of Engineering, International Islamic University Malaysia in the year 2007.

Callus and elicitors: Flowers of *C. indicum* were obtained from *C. indicum* farm in Cameron Highland, Pahang, Malaysia, latitude: 4 29' 00" and longitude: 101 27' 00". Chitosan extracted from crab-shell [M_r ~ 400000] was purchased from SIGMA, Malaysia, yeast extract from Difco and *A. niger* strain A103 from lab stock of the Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia.

***Chrysanthemum indicum* callus induction:** *Chrysanthemum indicum* callus was induced by culturing surface sterilized flower explants of *C. indicum* on MS (Murashige and Skoog) semisolid media (8 g L^{-1} plant agar, Phytotech) supplemented with 0.5 mg L^{-1} 6-BA(6-benzylaminopurine) and 2.0 mg L^{-1} NAA (naphthalene acetic acid). They were cultured for 3 weeks in dark conditions at 25°C.

***Chrysanthemum indicum* cell suspension culture preparation and establishment of the growth curve:** Initially, about 1.0 g fresh *C. indicum* callus, grown on MS semisolid media, were cut into small pieces and sub cultured on the same fresh MS media for proliferation. The *C. indicum* callus, weighing about 0.5 g, was then gently cut using forceps and blades into 20-30 small pieces and transferred into 100 mL MS liquid media. Three replicated samples were then prepared. The Erlenmeyer flask containing cell suspension culture of *C. indicum* callus was cultured on a rotary shaker set at 125 rpm and 25°C temperature.

The *C. indicum* cell suspension culture was then sub-cultured every week. Once the suspension culture become established having finely dispersed cell clusters and aggregates, a dilution ratio of 1:10 old culture to fresh medium could be possible on 7-10 days basis to maintain the cell-line. The well established cell suspensions were used as inoculums for the elicitor treatment.

Experimental design: Design of the experiment (DOE) and statistical analysis were done using statistical software, STATISTICA 6.0. The optimization study was conducted using general full factorial design with two factors and three levels (Table 1). The numbers of runs were 9 and average of triplicate readings for all the runs was recorded for accuracy. The selected factors were elicitor treatment time (day) and concentration of elicitors (g L^{-1}). Same DOE was used for all selected elicitors of chitosan, *A. niger* and yeast. The xanthine oxidase inhibition percentage was taken as the dependent variable or response (Y). A second order polynomial equation was then fitted to the data by multiple regression procedure. This resulted in an empirical model that related the response measured in the independent variables to the experiment. For a two-factor system, the model equation is:

$$Y = f(A, B)$$

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} AB \quad (1)$$

where, Y is the XO inhibitor activity (%), β_0, intercept; β_1, β_2, linear coefficients; β_{11}, β_{22}, squared coefficient and β_{12}, interaction coefficient.

Elicitor preparation and treatment: Chitosan, *A. niger* and yeast were bought and prepared beforehand. Percentage inhibition for control samples were also recorded for comparing the improvement in XO inhibition activity after the elicitor treatment (Table 2).

Table 1: Design of experiment for elicitor treatment using different elicitors

Run	Treatment time (day)	Elicitor concentration (g L^{-1})
1	-1	-1
2	-1	0
3	-1	1
4	0	-1
5	0	0
6	0	1
7	1	-1
8	1	0

Table 2: Percentage inhibition for control samples

Sample	OD reading (290 nm)	Inhibition (%)
Control 1-day 1*	0.758	42.424
Control 2-day 3*	0.796	43.939
Control 3-day 6*	0.798	48.485
Allopurinol	0.786	93.750
Intact flower	0.787	62.500

*Sample for control 1-3 was taken from cell suspension culture of day 1, 3 and 6, which are not treated with elicitor

Chitosan was prepared as suggested by Popp *et al*. (1997). Chitosan was dissolved in 5% (v/v) 1N hydrochloric acid (HCl) through gentle heating and continuous stirring. pH was adjusted to 5 with 1N sodium hydroxide (NaOH) and the final concentration was adjusted to 10 mg mL^{-1}. The solution was stirred to dissolve chitosan and then autoclaved for 15 min at 121°C. Finally, the solution was kept at 4°C prior to use. The concentrations for elicitation process prepared were 0.05, 0.10 and 0.25 g L^{-1} as stated in Table 3.

The medium for growing *A. niger* spores (30 g L^{-1} glucose, 2.5 g L^{-1} NH_4NO_3, 1.0 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $MgSO_4.7H_2O$, 0.05 g L^{-1} $ZnSO_4.7H_2O$) was prepared and sterilized by autoclaving for 15 min at 121°C. Inoculums were prepared (spore suspension) according to the method suggested by Jamal *et al*. (2005). Cultures grown at 32°C for 7 days was transferred into Erlenmeyer flask (250 mL) containing 100 mL of sterile distilled water. The flasks were shaken in a rotary shaker at 150 rpm for 24 h. The suspended fungal cultures were filtered using Whatmann No. 1 filter paper. Finally the filtrate was used as inoculums. For the preparation of the fungal elicitors, about 15 mL of *A. niger* inoculums were transferred into 500 mL flask containing 150 mL medium and was then cultured at 30°C at 150 rpm on a rotary shaker. The *A. niger* was harvested after 7 days of cultivation using Whatmann No. 1 filter paper. It was then dried in an oven at 90°C for 24 h and then grinded using mortar and pestle. The dried *A. niger* powder was measured and dissolved in distilled water to obtain concentration of 10 g L^{-1}. The mixture was then autoclaved at 121°C for 15 min before being added to the plant cell-culture. Finally, *A. niger* was prepared according to the desired concentration as shown in Table 4.

Ten gram yeast was dissolved in 100 mL of double distilled water and ethanol was added up to 80% (v/v) and kept at 4°C for 3 days for precipitation. The supernatant was then decanted and the precipitate was dissolved in 100 mL of double distilled water and then steam sterilized using autoclave for 15 min at 121°C. After sterilization, the YE elicitor was stored in a chiller before using it for elicitor treatment. The concentrations for elicitation process selected were 0.5, 1.0 and 1.5 g L^{-1} as stated in Table 5.

About 100 mL *C. indicum* suspension cells grown in 250 mL Erlenmeyer flasks were treated with three different concentrations of chitosan, *A. niger* and YE. Varying concentrations of elicitors were added to the cultures during day 1 (lag phase), day 3 (log phase) and day 6 (stationery phase) of growth as stated in Table 2, 4 and 6. After the elicitation, the cultures were maintained at 25°C in the dark for four days. Thereafter, the cells were filtered using Whatmann No. 93 filter paper. All experiments were carried out in triplicate and an average was taken in order to get an accurate result. Control sample was not elicited with any elicitor.

Table 3: Experimental and predicted percentage of XO inhibition when treated with chitosan elicitor

	Elicitor: Chitosan				
Samples	Treatment time (day)	Elicitor concentration (g L^{-1})	Experimental inhibition of XO (%)	Percentage inhibition increase (Fold)	Predicted inhibition of XO (%)
CH(A0)	1	0.05	34.848	0.821	30.513
CH(B0)	1	0.10	61.818	1.457	51.810
CH(C0)	1	0.25	25.758	0.607	40.253
CH(A3)	3	0.05	16.667	0.379	30.153
CH(B3)	3	0.10	40.909	0.931	49.570
CH(C3)	3	0.25	54.242	1.234	32.373
CH(A6)	6	0.05	12.121	0.250	3.213
CH(B6)	6	0.10	18.182	0.375	19.810
CH(C6)	6	0.25	-13.636	-0.281	-5.847

Table 4: Experimental and predicted percentage of XO inhibition when treated with *A. niger* elicitor

	Elicitor: *Aspergilus niger*				
Samples	Treatment time (day)	Elicitor concentration (g L^{-1})	Experimental inhibition of XO (%)	Percentage inhibition increase (Fold)	Predicted inhibition of XO (%)
AN(A0)	1	0.5	46.970	1.107	51.715
AN(B0)	1	1.0	31.212	0.736	32.950
AN(C0)	1	1.5	61.152	1.441	55.435
AN(A3)	3	0.5	62.121	1.414	58.275
AN(B3)	3	1.0	52.030	1.184	42.230
AN(C3)	3	1.5	53.333	1.214	67.435
AN(A6)	6	0.5	34.848	0.719	34.065
AN(B6)	6	1.0	13.636	0.281	22.100
AN(C6)	6	1.5	59.091	1.219	51.385

Table 5: Experimental and predicted percentages of XO inhibition when treated with yeast elicitor

	Elicitor: Yeast extract				
Samples	Treatment time (day)	Elicitor concentration (g L^{-1})	Experimental inhibition of XO (%)	Percentage inhibition increase (Fold)	Predicted inhibition of XO (%)
YE(A0)	1	0.5	50.000	1.179	42.330
YE(B0)	1	1.0	43.939	1.036	52.580
YE(C0)	1	1.5	37.879	0.893	37.800
YE(A3)	3	0.5	21.212	0.483	31.420
YE(B3)	3	1.0	60.606	1.379	51.520
YE(C3)	3	1.5	46.970	1.069	45.870
YE(A6)	6	0.5	13.636	0.281	11.005
YE(B6)	6	1.0	45.455	0.938	45.880
YE(C6)	6	1.5	53.030	1.094	55.005

Table 6: ANOVA for the selected quadratic model (Chitosan)

Source	df	Sum of squares	Mean squares	F-value	p>F
Regression	5	3054.5	610.9	1.52	0.388
Residual error	3	1206.7	402.2		
Total	8	4261.3			

The flirted cells were weighed after drying (at 60°C for 24 h) and grinding. The active compounds were extracted from the dried cells. The samples were kept in tubes and 1.5 mL of distilled water was added to each samples. The extraction process was carried out using water bath at 30°C for 16 h. The extracts were then diluted with phosphate buffer to make a concentration of 100 µg mL^{-1} (test solution).

XO inhibitor activity measurement: The mixture was consisted of 0.3 mL of 0.1 M phosphate buffer (pH 7.5), 0.1 mL of test solution (100 µg mL^{-1}), 0.1 mL distilled water and 0.1 mL of 0.12 U mL^{-1} xanthine oxidase. The mixture was incubated at 25°C for 15 min and after that, the reaction was initiated by the addition of 0.2 mL of 150 mM xanthine substrate solution. The test mixture was incubated again at 25°C for 30 min. The reaction was stopped by adding 0.2 mL 1 N HCl. The uric acid produced was monitored at 290 nm using a UV-spectrophotometer. The values obtained were the mean of the three replications of the sample. Allopurinol (100 µg mL^{-1}) was used as positive control. XO inhibitory activity was expressed as the percentage inhibition of XO in the above assay system, calculated as:

$$(1-B/A) \times 100$$

where, A and B are the activities of the enzyme without and with test material.

RESULTS AND DISCUSSION

Effect of chitosan, *A. niger* and yeast extract on XO inhibitor activity: Data obtained from this experiment (Table 1-7) shows that chitosan is a potential elicitor that can enhance XO inhibitor activity compared to other elicitors tested. It gave inhibition percentage of 61.818% (Table 3) as compared to control (42.424% in Table 2) inhibition and this has been 1.457 fold higher than the control sample (Table 3) when treated using a concentration of 0.1 g L^{-1} on day 1. Although many other finding showed that elicitation is efficient at late log phase but our finding was contradict. This might suggests that the elicitation stress alone is not sufficient to enhance the productivity. It needed the inoculation stress together with elicitation stress to enhance the productivity of XOI in *C. indicum* cell suspensione (Lu *et al*., 2001). However, this percentage is still quite low as compared to allopurinol (positive control) percentage of inhibition. Nevertheless, as compared to the intact plant extracts (62.500% inhibition, Table 2), the activity is nearly the same. Moreover, from the results obtained, it can be concluded that elicitation using chitosan has fairly near to the value of intact plant of *C. indicum*. From the study done by Kong *et al.* (2000), it is evident that ethanol extracts of *C. indicum* has inhibited 95% XO using the same concentration (100 µg mL^{-1}), whereby water extracts can only inhibit 68% XO. This value is closer close to the value obtained from this study, which is 62.5% for intact plant of *C. indicum* at the same concentration. It can, thus, be concluded that the low percentage inhibition obtained for this study was due to water extraction and a higher percentage might be obtained if other type of solvent system was used.

Table 7: Results of regression analysis of the full factorial design (Chitosan)

Predictor	Coefficient	Standard error coefficient	t-value	p-value
Constant	-9.420	45.090	-0.21	0.848
X_1	7.790	18.070	0.43	0.696
X_2	821.900	631.200	1.30	0.284
X_1X_1	-1.761	2.379	-0.74	0.513
X_1X_2	-18.840	38.280	-0.49	0.656
X_2X_2	-2515.000	1968.000	-1.28	0.291

S = 20.06; R^2 = 71.7%; R^2(adj) = 24.5%

The highest inhibition of *A. niger* was 62.121% (Table 5) and 60.606% for yeast (Table 6), but the treatment time was at day 3, which is much longer than chitosan treatment. The results prove that treatment with elicitor especially chitosan can be used as a better alternative to overcome the problem of lower yield in cell suspension culture system. Some encouraging results were also observed by Zhang *et al.* (2007), when the *Taxus chinensis* cell suspension culture adapted to chitosan gave 3.2 fold yields and proved that treatment with chitosan is an effective strategy for improving yield.

Chitosan is the deacetylated form of chitin, which is the main component of the cell-walls of some fungal species and of the exoskeletons of insects and crustaceans, being the second most abundantly available natural polysaccharide on the earth, just next to cellulose. Consequently, chitosan has been widely applied as a potent elicitor in plant cell suspension cultures to enhance secondary metabolite production (Kim *et al*., 1997). Chitosan, as an effective oligosaccharide elicitor, has also recently been proved to significantly improve accumulation of secondary metabolites in other plant cell cultures (Kim *et al*., 1997; Komariah *et al*., 2002). Addition of chitosan elicitor dramatically stimulated PeGs (phenylethanoid glycosides) biosynthesis in *C. deserticola* cell-culture, when the stimulation was associated with PAL (L-phenylalanine ammonia-lyase), the first key enzyme in PEGs biosynthesis (Cheng *et al*., 2005). Optimum incubation time was 48 h for obtaining the highest level of metabolite with chitosan and *A. niger* (Komariah *et al*., 2002).

However, from all the results obtained (Table 3-5), chitosan gave the highest experimental value. The data obtained was further analyzed and statistical optimization was done using Statistica software.

Statistical analysis results on elicitation: The regression equation, analysis of result, construction of surface and contour plots were obtained using statistical software, which eventually assisted in determining the optimum treatment time as well as optimum concentration of elicitor to obtain the highest inhibition activity of XO. The statistical optimization predicted the highest inhibition activity of 60.975% at 1.25 day or 30 h culture time using a concentration of 0.16 g L^{-1} of chitosan.

The fungal elicitor, *Aspergillus niger*, demonstrated the maximum inhibition at its highest concentration of elicitor on day 1 of cultivation. The percentage inhibition of xanthine oxidase increased by 1.441 fold compared to control sample. However, the predicted value obtained by the software was 0.15 g L^{-1} of elicitor concentration and the highest inhibition of XO was obtained on 3rd day. The predicted value was 67.435% and the experimental value obtained was 53.333%. It can be noticed that the difference between the predicted and actual value was quite high. On the other hand, inhibitory effects were quite low on day 6 of cultivation, except for 0.15 g L^{-1} concentration. Compared to sample from intact plant, it can be concluded that the value attained by inhibition of XO by *A. niger* is closer to the value of intact plant of *C. indicum*. However, fold increment was still low as compared to chitosan elicitation.

The YE elicitor, induced 1.379 fold percentage inhibition of xanthine oxidase activity over control sample using 1.5 g L^{-1} concentration on day 3 of elicitation (Table 5), which was the highest inhibition attained by YE. The predicted value of highest percentage inhibition of XO (55.005%) obtained after analysis by the software was 0.15 g L^{-1} of concentration and elicitation time of 6 days, which is relatively undesirable considering cost and time consumption (Table 5). Perhaps for this reason, Repka (2001) has suggested that it was possible that different elicitors induced different parts of the defense response. Others may have synergistic effects on the same pathway. Nevertheless, the host signal molecules and the mechanisms underlying both elicitor perception at the plant cell surface and subsequent intracellular transmission of this signal to target sites are still not fully understood. Therefore, it is hard to explain the underlying mechanism inhibition obtained by *A. niger*, chitosan and YE.

Regression analysis was performed to fit the response function with the experimental data. In this study, the coefficient of variation obtained were: $R^2 = 0.71$ for chitosan, $R^2 = 0.76$ for *A. niger* and $R^2 = 0.81$ for YE, indicating a relatively high correlation between the experimentally observed and predicted values. It indicates the degree of precision with which the secondary metabolite (xanthine oxidase inhibitor) production is attributed to the independent variables, treatment time and elicitor concentration. Results clearly indicate that 76.7, 81.7 and 71.7% of the variation in inhibition activities elicited by *A. niger*, YE and chitosan respectively is explained by the model. The polynomial regression Eq. 2-4 were obtained on XO percentage inhibition using chitosan, *A. niger*, YE, respectively. The experimental and predicted values for all elicitors are given in Table 3-5:

$$\text{Inhibition XO (\%)} = -9.4 + 7.8 X_1 + 822 X_2 - 1.76 X_1X_1 - 18.8 X_1X_2 - 2515 X_2X_2 \quad (2)$$

$$\text{Inhibition XO (\%)} = 103 + 11.0\,X_1 - 164\,X_2 - 2.27\,X_1X_1 + 2.72\,X_1X_2 + 82.5\,X_2X_2 \quad (3)$$

$$\text{Inhibition XO (\%)} = 15.9 - 9.3\,X_1 + 87.9\,X_2 - 0.27\,X_1X_1 + 9.85\,X_1X_2 - 51.5\,X_2X_2 \quad (4)$$

The corresponding Analysis of Variance (ANOVA) for chitosan is presented in Table 6. The computed F-value (1.52) indicates the significance of the model at high confidence level. The probability p-value was also relatively low $[(p_{model} > F) = 0.388)$, which indicates that the model has significant explanatory power or the model is good.

Based on the regression analysis for the elicitors, the t-distribution and the corresponding p-values of the variables along with the second order polynomial coefficient were evaluated (Table 7). The significance of each factor was determined by these values because the pattern of interaction between the factors is indicated by these coefficients. The large magnitude of t-test and small p-values (less than 0.05) indicate the high significance of corresponding coefficient. The variables with low probability value contribute to the model; whereas, variables with high values can be eliminated from the model. In the Table 7, it can be observed that the chitosan concentration is the most significant contributing factor. This indicates that it can act as a limiting factor or a nutrient and little variation in its concentration will alter the percentage inhibition of XO.

Surface response and contour plot: The surface response analysis (Fig. 1) has estimated the optimum inhibition of xanthine oxidase over independent variables, treatment time (X_1) and elicitor concentration (X_2). The main objective of response surface is to determine optimum values of the variables efficiently in order to obtain maximized response. Each contour curve represents an infinitive number of combinations of two-test variables. The highest predicted value indicated by surface confined in the smallest ellipse in the contour diagram. Elliptical contours were obtained when there was a perfect interaction between independent variables.

The red elliptical shades from contour plot analysis (Fig. 2) in the middle of the quadratic curve characterize the optimum area of interaction between the two parameters to show the maximum percentage inhibition of

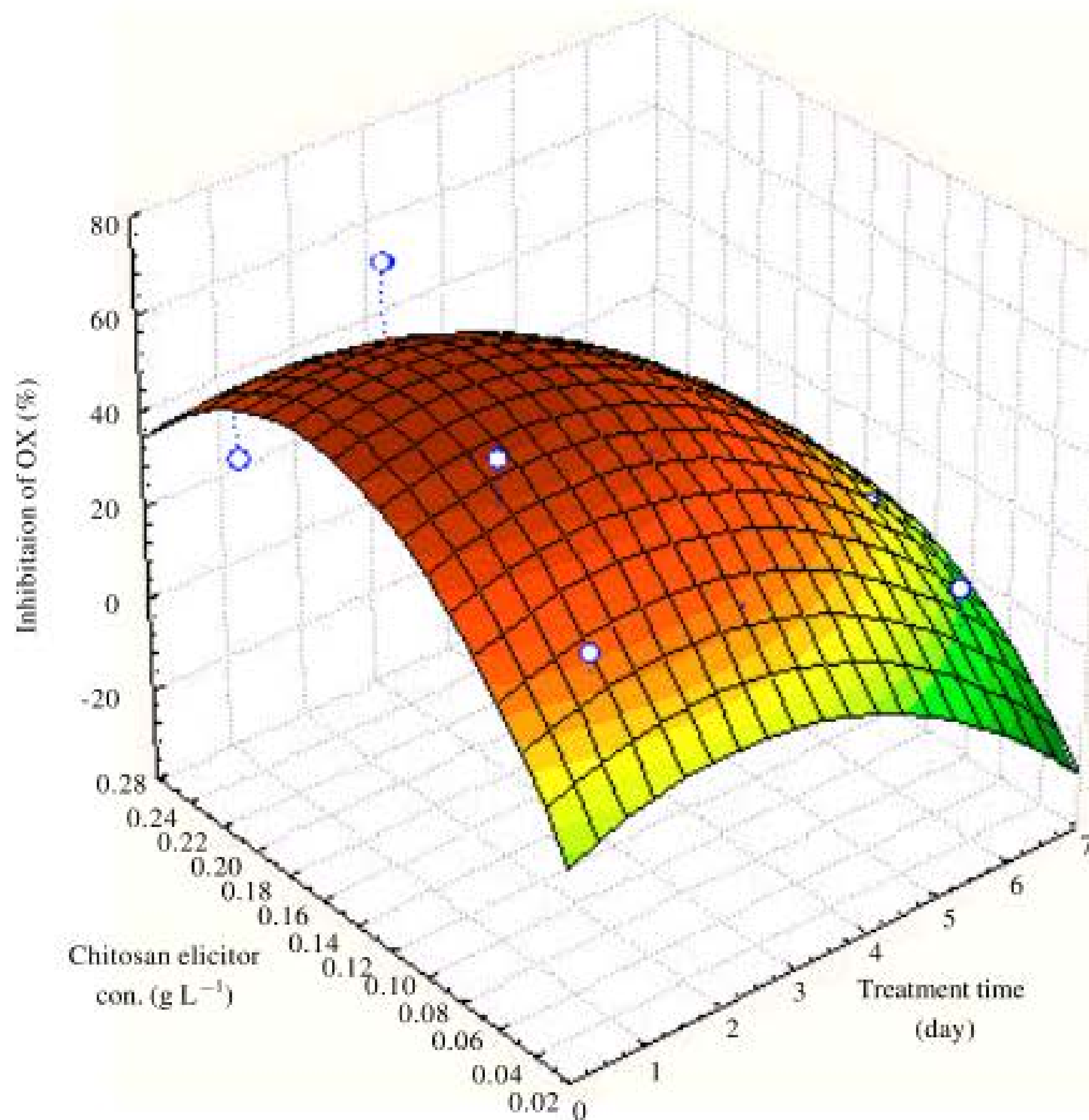

Fig. 1: Surface plot for percentage inhibition of XO as a function of chitosan concentration and treatment time

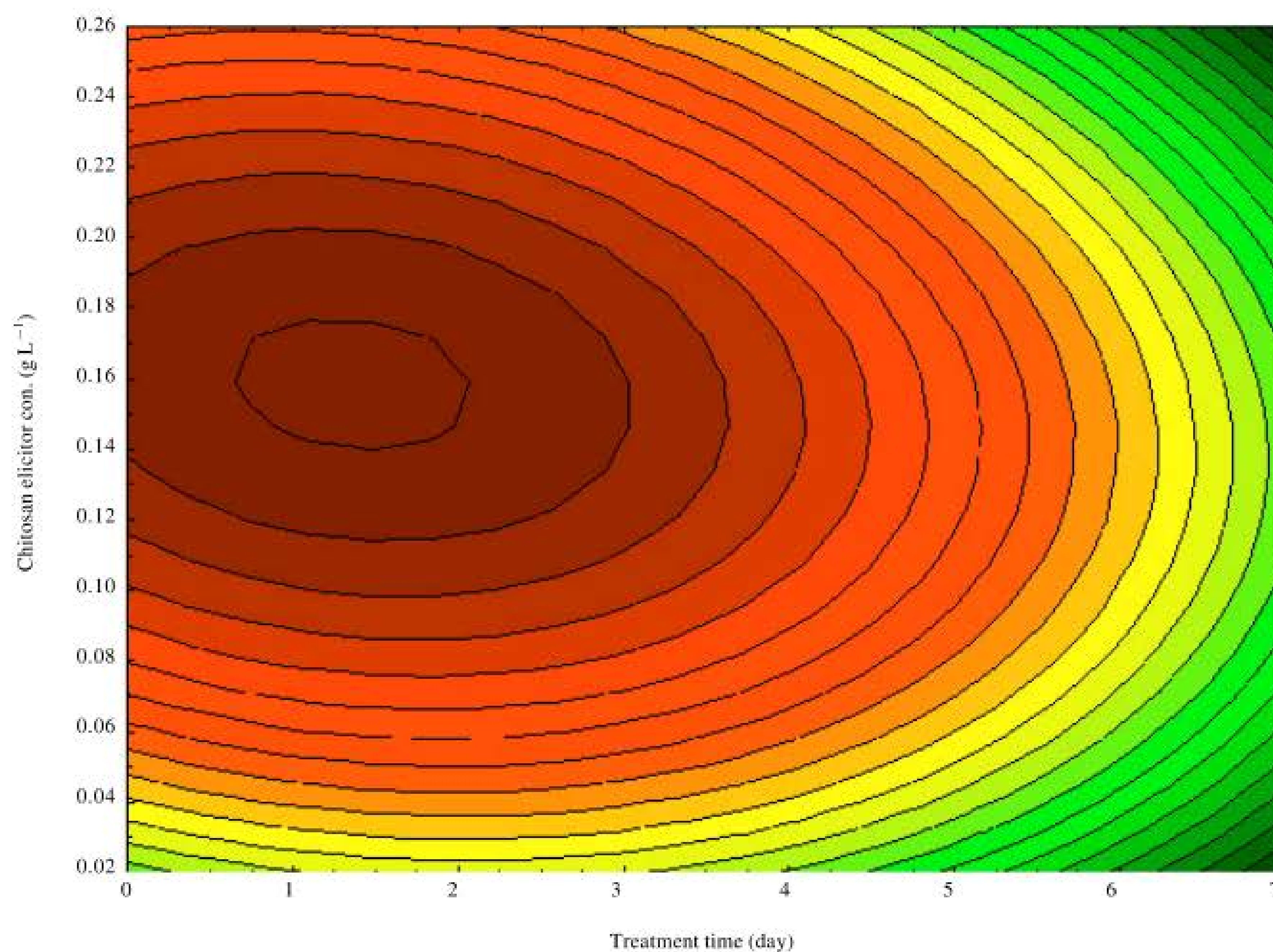

Fig. 2: Contour plot for percentage inhibition of XO as a functiron of chitosan concentration and treatment time

XO. Thus, the maximum percentage inhibition of XO that can be achieved is 60.975% when elicited at day 1.25 or 30 h at concentration of 0.16g L^{-1} of chitosan.

CONCLUSION

In conclusion, chitosan is the most suitable elicitor for XO inhibitor production from *C. indicum*. The optimum concentration of chitosan required is 0.16 g L^{-1} and the optimum time for elicitor treatment is day 1.25 or 30 h of cultivation to enhance percentage inhibition of XO. This conclusion was drawn on the basis of the recorded data showing an increase of 1.457 fold inhibition activity compared to the control sample after being elicited by chitosan at the optimum conditions. *A. niger* and YE also exhibit significant effect in inducing enhancement of XO inhibitor activity. However, chitosan is the best elicitor and has successfully enhanced the inhibition activity.

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