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إستخدام التصميم الإحصائي Central Composite Design للوصول إلي الظروف المثلي لإنتاج الحمض ١٨ بيتا جليسرهيتنك بإستخدام الخميرة تريكوسبورون جيروفيسياي

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الملخص العربي

في دراسة سابقة تم إستخدام تصميم Plackett-Burman لتحديد أفضل العوامل المؤثرة في إنتاج حمض الجليسرهيتينك من الجليسرهيزين المستخلص من جذور نبات العرق سوس بإستخدام الخميرة تريكوسبورون جيروفيسياي، أما في هذة الدراسة تم إستخدام تصميم Central Composed لتحديد العوامل المثلي للإنتاج. المتغيرات المستقلة المستخدمة هي درجة الحموضة، فترة التحضين، تركيز الجلوكوز، تركيز مستخلص الخميرة و مستخلص الذرة. ووجد أن التصميم Central Composed قادرعلي التنبؤ بأثر المتغيرات المستقلة علي الإستجابة. تم إستخدام تحليل التباين لتحديد مدي ملائمة التصميم المستخدم والقيمة التجريبية، وأشارت النتائج إلي أن هذا النموذج كان شبة كاف ومناسب إلي حد ما مع البيانات الفعلية وكانت قيمة r التربيعية هي (0.548). وكان أعلي تركيز متحصل علية هو ١٥٨ ملجم لكل ٠.٦ جرام من المستخلص الخام للجليسرهيزين.

CENTRAL COMPOSITE DESIGN FOR THE OPTIMIZATION OF 18B-GLYCYRRHETINIC ACID PRODUCTION BY *TRICHOSPORON JIROVECII*

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ABSTRACT: *In our previous study Plackett-Burman design was used to select the most effective variables for a high production of 18β-glycyrrhetinic acid (GA). In this work the Central Composite Design (CCD) combined with Response Surface Methodology (RSM) were used to optimize the conditions for GA production from glycyrrhizin (GL) by the yeast Trichosporon jirovecii. The used independent variables were initial pH, incubation time, glucose, yeast extract and corn steep liquor. A mathematical model that is able to predict the effect of independent variables towards the responses was established by multiple regression analysis. Analysis of variance was used to determine the adequacy between the model and experimental value. The results indicated that the model was semi-adequate with satisfactory of the RSquare (0.548). It was fairly good experimental model fits the actual data. Using this design a high conc. (158 mg) of GA was produced from 0.6 g of crude GL.*

Key words: *Central Composite Design, Response Surface Methodology, Trichosporon jirovecii, glycyrrhizin, 18β-glycyrrhetinic acid.*

INTRODUCTION

Licorice root (*Glycyrrhiza glabra*) has been used in both Eastern and Western medicine to treat a variety of illnesses ranging from the common cold to liver disease. This herb has long been valued as a demulcent (soothing, coating agent) and expectorant (rids phlegm and mucous from the respiratory tract) and continues to be used by health care professionals today to relieve respiratory ailments (such as allergies, bronchitis, colds, sore throats, and tuberculosis), stomach problems (including heartburn from reflux), inflammatory disorders, skin diseases, stress relief, and liver problems Borrelli and Izzo (2000).

Glycyrrhizin (GL) is a triterpenoid saponin glycoside (4-20%), a mixture of potassium and calcium salts of glycyrrhizic acid (or glycyrrhizinic) acid. Upon hydrolysis, the glycoside loses its sweet taste and is converted to the aglycone 18-β-glycyrrhetinic acid (Fig. 1), (a pentacyclic triterpine carboxylic; GA) plus two molecules of glucuronic acid. Glycyrrhizin is the active principle of licorice root. It is a powerful sweetener, which is 50 times as potent as sucrose (cane sugar) Akao and Hattori (1991).

GL, which is classified as a saponin, has a protective effect against hemolysis induced by other saponins or a cationic surfactant.

GA and its derivatives are mainly applied in pharmaceutical and cosmetic fields for its multiple functions of have anti-bacteria, antioxidant, anti-mutation, anti tumer, anti-inflammatory, anti-arthritic properties and anti-tussive activity Borrelli and Izzo (2000).

GA can reduce serum cholesterol levels and it also inhibits the growth of several DNA and RNA viruses, inactivating herpes simplex virus particles irreversibly.

Both GA and DGL are effective in treating ulcers. The effect is not due simply to the chemicals' anti-inflammatory properties, but also probably involves their ability to inhibit gastric acid secretion.

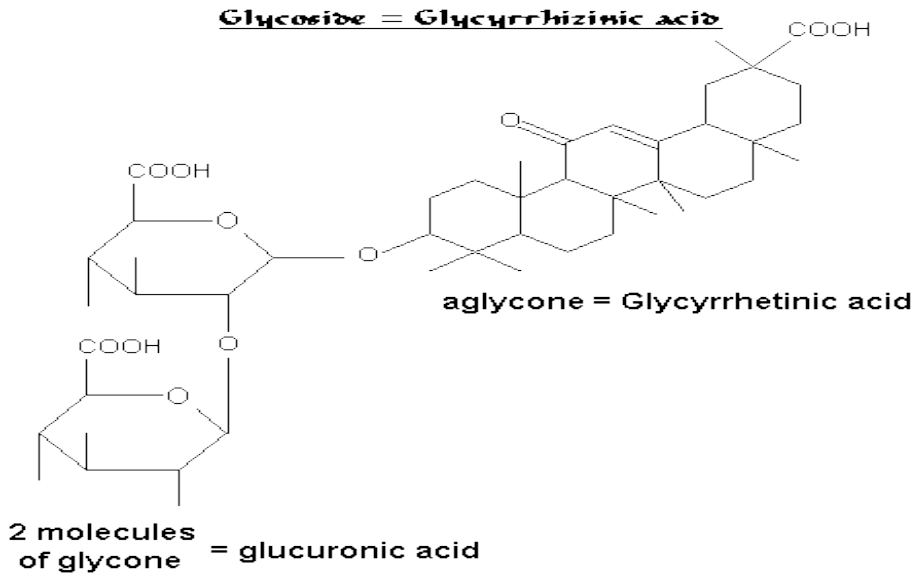

Figure 1: Chemical structures of glycyrrhizinic acid and glycyrrhetinc acid.

GA had an anti-hyperglycemic effect, which is evidenced by lowered plasma glucose with a simultaneous increase in the insulin secretion. 100 mg/kg/body weight showed promising anti-hyperglycemic effect, and is comparable to glibenclamide Kalaiarasi and Pugalendi (2009).

In the circulatory system, licorice root and/or its derivatives have recently demonstrated exciting results as interferon inducers in the immune system. GA holds great promise in the treatment of liver diseases such as hepatitis and cirrhosis.

Stronger Neo-minophagen C (SNMC) is an intravenous solution composed of GL (0.2%), cysteine (0.1%), and glycine (2.0%) has been used widely and successfully in Japan as an anti-hepatitis drug. When chronic active hepatitis is treated with SNMC, 40 ml daily for 4 weeks, it accelerates the improvement of serum transaminase; prompt improvement with high statistical significance has been observed, compared with those who received a placebo Van Rossum *et al*. (2001).

In the United States, GL is classified as "generally recognized as safe" a flavoring agent, although not as a sweetener. GL is used as a flavoring in some candies, pharmaceuticals, and tobacco products. The European Union suggests that people should not consume any more than 100 mg of glycyrrhizic acid a day European Commission (2003), equivalent to approximately 50 g of licorice sweets Størmer *et al*. (1993).

The most widely reported side effects of GL use are hypertension and edema (water retention). These effects are related to the inhibition of cortisol metabolism within the kidney, and the subsequent stimulation of the mineralocorticoid receptors. Thus, consumption of black licorice can mimic disorders of excess aldosterone Ferrari *et al*. (2001).

The content of GA in licorice is much less than that of GL. In general, the bioactivity of the aglycon is much stronger than that of glycoside Yu *et al*. (1999). GA is responsible for the pharmacological effects when licorice is applied Hansen *et al*. (1999). To reach the same therapeutic effectiveness, the dosage of GA administrated alone is much less than that of GL. For example, during the prevention of liver cancer cells from proliferating and the induction of its polarization and reversion, the dosage of GL

is 20 times as that of GA Ge *et al*. (1999). Accordingly, GA is much preferred to GL Sun *et al*. (2010).

Optimization of production conditions is an important problem in the development of economically feasible bioprocesses. Combinatorial interactions of process variables with the production of the desired compound are numerous and the optimum processes may be developed using an effective experimental design procedure. Response Surface Methodology (RSM), which is a collection of statistical techniques for manipulative experiments, building models, evaluating the effects of factors and searching for the optimal conditions, has successfully been used in the optimization of bioprocesses Muthuvelayudham and Viruthagiri (2010).

The main target of this study is to apply Central Composite Design (CCD) based Response Surface Methodology (RSM) to analyze the effects of the process parameters on GA production from GL by yeast cells *Trichosporon jirovecii*.

MATERIALS AND METHODS

1. Chemicals:

GL (glycyrrhizic acid ammonium salt) ≥ 95.0 % (*Biochemika*), GA 97.0 % (*Aldrich*) and ρ-nirtophenyl glucuronide (*Sigma*) were purchased from Sigma-Aldrich Chemical Co.

All other chemicals were of analytical reagent grade.

2. Source of plant:

Plant roots (Licorice) were collected from the Medicinal and Aromatic Plants Research department, Horticulture Research Institute (HRI), Agriculture Research Center, Ministry of Agriculture, Gize, Egypt; at the normal harvesting time. Roots were washed, dried and ground, then stored at 4˚C until extraction.

3. Microorganism:

The yeast used in this study is *Trichosporon jirovecii* which is previously isolated and identified Shetaia *et al*. (2005). This strain was selected to test its biotransformation ability for GL into GA.

4. Growth media:

4.1. Maintenance medium:

Yeast-malt agar (YMA) medium, which has the following composition expressed as a percentage (g/100 ml): yeast extract 0.3; malt extract 0.3; glucose anhydrous 1.0; agar 1.5, the final pH (at 25˚C) was adjusted to 6.2±0.2.

4.2. Yeast cultivation:

The inoculum was prepared by scratching with a sterile needle from YMA slant into 10 ml of Yeast Malt Broth (YM Borth). The suspension was used to inoculate 100 ml of sterile GL fermentation medium dispensed in a 250 ml Erlenmeyer flask.

Flasks were then incubated on a rotary shaker with 120 rpm for different periods of incubation and at certain temperature, according to experimental design in Table (1).

After that, cultures were centrifuged at 6000 rpm for 15 min to separate the yeast cells from the culture filtrate. pH, yeast biomass, glucose content, protein content, enzyme activity and GA content were then determined.

5. Extraction of GL from Licorice Root:

GL was extracted from licorice roots according to the method of Hartung and Harold (1979).

6. Analytical methods for Biotransformation of GL into GA:

6.1. Glucose, protein and biomass determination:

Glucose, protein and biomass were determined as previously described El-Baz (2012).

Table (1): Factors and coded levels examined as independent variables affecting GA production by *Trichosporon jirovecii* and their levels in the Central Composite Design experiment.

Independent variables	Units	Experimental values Coded levels		
		-1	0	1
Initial pH	pH	6.0	6.5	7.0
Incubation time	day	7	8	9
Glucose (%)	g/100 ml	0.0	0.5	1.0
Yeast extract (%)	g/100 ml	0.1	0.2	0.3
C.S.L. (%)	g/100 ml	0.8	1.0	1.2

6.2. Isolation and determination of GA:

GA was extracted from fermented media by ethyl acetate and concentrated to a small volume. GA was determined qualitatively on TLC plate and quantitatively using HPLC Tanaka *et al*. (1990).

6.3. β-glucuronidase activity assay:

The β-glucuronidase activity of samples was measured by ρ-nirtophenyl glucuronide. A unit of β-glucuronidase activity was expressed as the amount of enzyme which liberated 1μmol of ρ-nirtophenyl/min during the hydrolysis reaction Szasz (1967).

7. Experimental designs:

7.1. The Central Composed Design (CCD):

The Central Composed experimental design, a fractional factorial design, was used to reveal the relative importance of various environmental factors on GA production in liquid cultures. Five independent variables were screened in 32 runs organized according to the Central Composed Design matrix (Table 2). In this model, the most significant factors or independent variables are included initial pH, incubation time, glucose, yeast extract and corn steep liquor and each independent variable can be tested at the three different levels, low (-), high (+) and central or basal (0).

The other following conditions were used at levels as concluded from our previous work El-Baz (2012); inducible concentration 0.6 %; inducible addition time 20 hrs; aeration 1/10 and temperature 30˚C, in addition to, $NaNO_3$ 0.3%; $MgSo_4.7H_2O$ 0.05% and K_2HPO_4 0.1 %.

All trials were performed in duplicates in 250 ml Erlenmeyer flasks and final data was the mean of the duplicate data. High, low and basal levels of independent variables have been shown in Table (1). The averages of GA production results were treated as the responses.

7.2. Statistical Analysis

The optimization of cultivation conditions was an important problem in the development of economically feasible bioprocesses. Combined interactions of medium parameters for the production of the desired product are large and the optimal process conditions may be industrial using an effective experimental design procedure. Response Surface

Methodology (RSM), which is a collection of statistical techniques for design of experiments, building models, evaluating the effects of factors and searching for the optimum conditions, has successfully been used in the optimization of bioprocess.

Table (2): The Central Composite Design for 5 variables.

Trial	pH	Incubation time	Glucose %	Yeast extract %	C.S.L.
1	7.0	7	0.0	0.1	1.2
2	7.0	7	1.0	0.3	1.2
3	6.5	8	0.5	0.2	1.0
4	6.5	8	0.5	0.2	0.8
5	6.0	7	0.0	0.3	1.2
6	6.0	8	0.5	0.2	1.0
7	7.0	9	0.0	0.3	1.2
8	6.5	8	0.5	0.1	1.0
9	7.0	9	1.0	0.1	1.2
10	6.5	8	0.5	0.2	1.0
11	6.0	9	0.0	0.1	1.2
12	6.5	8	0.5	0.3	1.0
13	6.5	7	0.5	0.2	1.0
14	7.0	7	1.0	0.1	0.8
15	6.5	8	0.5	0.2	1.0
16	6.0	9	1.0	0.1	0.8
17	6.0	7	0.0	0.1	0.8
18	7.0	7	0.0	0.3	0.8
19	6.5	8	0.5	0.2	1.0
20	6.5	8	0.5	0.2	1.2
21	6.5	8	0.5	0.2	1.0
22	6.0	9	0.0	0.3	0.8
23	6.0	7	1.0	0.3	0.8
24	6.5	9	0.5	0.2	1.0
25	7.0	9	0.0	0.1	0.8
26	6.5	8	0.5	0.2	1.0
27	6.5	8	1.0	0.2	1.0
28	6.0	9	1.0	0.3	1.2
29	6.5	8	0.0	0.2	1.0
30	6.0	7	1.0	0.1	1.2
31	7.0	8	0.5	0.2	1.0
32	7.0	9	1.0	0.3	0.8

N.D.[1] = Not Detected

The statistical analysis of the model was performed in the form of Analysis of Variance (ANOVA), this analysis included the Fisher's *F*-test, its associated probability *p* (F), correlation coefficient *R*, determination coefficient *R* square which measures the goodness of fit of regression model. Its also includes the Student's *t*-value for the estimated coefficient and associated probabilities *p*(t).

RESULTS AND DISCUSSION

1. Extraction of crude glycyrrhizin from licorice root:

Quantity of crude glycyrrhizic acid (as the fermentation inducible substrate) was 5.38 g per 100g ground licorice roots, with the purity of 64.44 % as estimated by HPLC.

2. Optimization of GA production by Central Composite Design:

Central composite design is an experimental design, useful in response surface methodology, for building a second order (quadratic) model for the response variable without needing to use a complete three-level factorial experiment. After the designed experiment is performed, linear regression is used, sometimes iteratively, to obtain results.

Optimization of parameters like initial pH, incubation time, glucose, yeast extract and corn steep liquor were made on cultivation process. Central Composite Design experiments using response surface methodology was proved to be most selective tool for optimization of medium parameters for GA production.

The levels of the independent variables (coded values of factors) were determined in this study based on preliminary experiments. The central values (zero level) chosen for experimental design were initial pH 6.5, incubation time (8 days), glucose (0.5 %), yeast extract (0.2 %) and corn steep liquor (1.0 %).

From pervious study using a Plackett-Burman Design pH (6), incubation time (7 days), glucose (1 %), yeast extract (0.3 %) and corn steep liquor (0.8 %) were selected as the most significant variables for the highest level of GA production, respectively. However, temperature (30°C), inducible (GL) concentration (0.6 %), inducible addition time (20 hrs) and aeration (25 ml/ 250 ml flask) were selected as the less significant factors.

The experimental values for GA production under different treatment conditions are presented in Table (3). pH, protein content, glucose content, biomass and enzyme activity were determined after 7, 8 and 9 days according to the proposed design. The highest GA accumulation record (158.0 mg) was achieved by the trial number 30.

The regression coefficients and results for the linear, quadratic and interaction term are presented in Table (4). The statistical analysis indicates that the model was semi-adequate, possessing comparatively significant lack of fit and with satisfactory of the RSquare (0.548). It was fairly good experimental model fits the actual data.

The decrease in the value of RSquare was due to the coded levels of factors that were identified; and were closed to a great extent, which led to not observe any difference between independent variables; In addition to contain coded levels of glucose central level zero.

The results of this experiment suggested most effective combination variables, concerning GA production according to statistical analyses of the data (*t* ratio).

Fig (2) shows Pareto Plot of the effects for experimental variables that were the interactions between the yeast extract with C.S.L., pH with yeast extract and incubation time with glucose.

The relation between incubation time and pH as independent variables; that affect the enzyme activity based on the results of response surface of the central composite design experiments is show in Fig (3). It is noted from the results that there is a direct correlation between enzyme activity and GA production by *Trichosporon jirovecii*.

Table (3): Results of parameters determined during GA Production by *Trichosporon jirovecii* using Central Composition Design (CCD).

Trial	Biomass %	pH	Glucose %	Protein %	Enzyme activity	GA
1	0.545[1]	9.13	N.D. [2]	0.812[3]	0.023*	6.18
2	1.715	8.88	N.D.	0.752	0.477	128.17
3	0.795	8.60	N.D.	1.525	0.122	32.78
4	0.710	8.53	N.D.	0.835	0.169	45.41
5	0.520	9.03	N.D.	0.843	0.087	23.38
6	0.860	8.70	N.D.	0.855	0.190	51.32
7	0.425	9.05	N.D.	0.013	0.000	N.D.[1]
8	0.980	8.59	N.D.	0.965	0.129	34.66
9	1.190	8.53	N.D.	0.090	0.265	71.21
10	0.795	8.72	N.D.	1.140	0.225	60.46
11	0.545	8.81	N.D.	0.029	0.007	1.90
12	0.735	8.72	N.D.	0.860	0.115	30.90
13	0.755	9.01	N.D.	0.729	0.224	60.19
14	1.530	8.48	N.D.	0.698	0.516	138.65
15	0.610	8.58	N.D.	0.835	0.242	65.03
16	0.860	8.19	N.D.	0.009	0.405	108.83
17	0.475	8.93	N.D.	0.577	0.080	21.50
18	0.510	9.04	N.D.	0.752	0.080	21.50
19	1.080	8.57	N.D.	0.750	0.249	66.91
20	0.760	8.79	N.D.	1.005	0.229	61.53
21	0.695	8.75	N.D.	1.465	0.244	65.56
22	0.645	8.62	N.D.	0.016	0.005	1.34
23	1.365	8.58	N.D.	0.843	0.573	153.97
24	0.835	8.69	N.D.	0.088	0.096	25.79
25	0.520	8.45	N.D.	0.081	0.000	N.D.
26	0.550	8.77	N.D.	0.885	0.213	57.23
27	1.140	8.40	N.D.	0.925	0.417	112.05
28	1.025	8.58	N.D.	0.037	0.398	106.94
29	0.435	8.89	N.D.	1.980	0.020	5.37
30	2.545	8.41	N.D.	0.683	0.588	158.00
31	0.745	8.79	N.D.	0.920	0.101	27.14
32	0.885	8.49	N.D.	0.069	0.389	104.53

[1]dry weight (g/ 100 ml)
[2]"N.D."= Not Detected
[3]% of total protein (g/100 m l)
*Unit of β-glucuronidase activity was expressed as the amount of enzyme which liberated 1μmol of ρ-nirtophenyl/min

Table (4): Statistical analysis of most effective combination of variables concerned with GA production.

Term	Estimate	Std Error	t-Ratio	Prob>|t|
Intercept	0.2276195	0.056078	4.06	0.0019*
incubation time(7,9)	-0.019167	0.046251	-0.41	0.6865
pH(6,7)	0.0268889	0.046251	0.58	0.5727
yeast extracts(0.1,0.3)	0.0237778	0.046251	0.51	0.6173
glucose(0,1)	0.0036667	0.046251	0.08	0.9382
CSL(0.8,1.2)	-0.065556	0.046251	-1.42	0.1841
incubation time*pH	-0.027875	0.049056	-0.57	0.5813
incubation time*yeast extracts	0.003	0.049056	0.06	0.9523
pH*yeast extracts	-0.085625	0.049056	-1.75	0.1087
incubation time*glucose	-0.055625	0.049056	-1.13	0.2809
pH*glucose	0.002	0.049056	0.04	0.9682
yeast extracts*glucose	-0.029625	0.049056	-0.60	0.5582
incubation time*CSL	0.009125	0.049056	0.19	0.8558
pH*CSL	-0.00125	0.049056	-0.03	0.9801
yeast extracts*CSL	-0.093875	0.049056	-1.91	0.0820
glucose*CSL	0.024	0.049056	0.49	0.6343
incubation time*incubation time	-0.034709	0.125088	-0.28	0.7866
pH*pH	0.1107908	0.125088	0.89	0.3947
yeast extracts*yeast extracts	0.0007908	0.125088	0.01	0.9951
glucose*glucose	0.0217908	0.125088	0.17	0.8649
CSL*CSL	-0.121209	0.125088	-0.97	0.3534

RSquare= 0.548

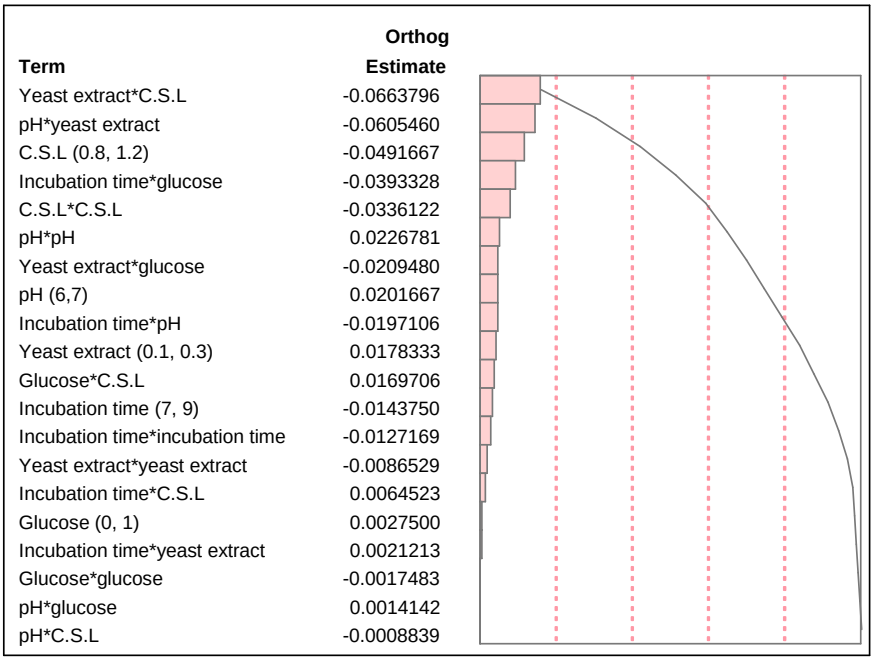

Figure (2): Pareto Plot of the effects for experimental variables.

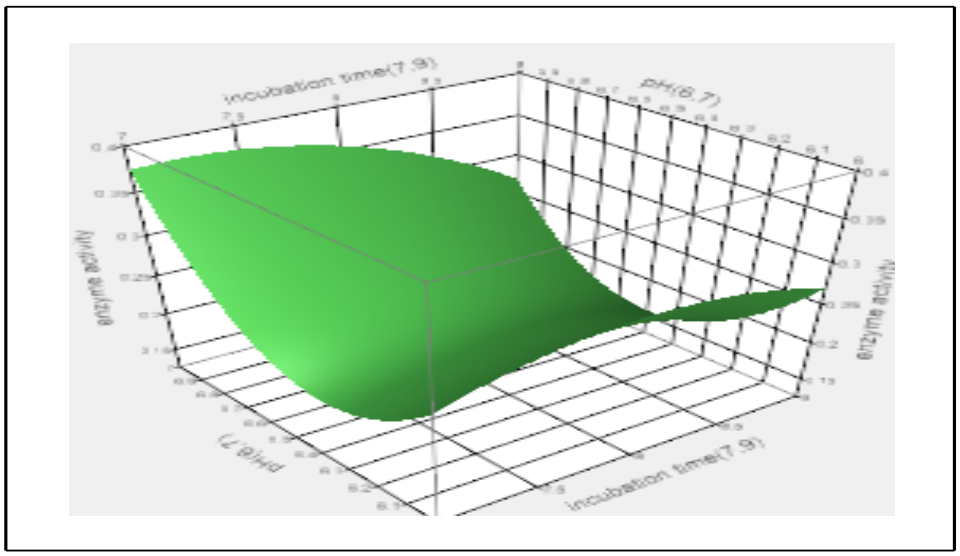

Figure (3): The Response surface for GA production using Central Composite Design (CCD) experiment.

It has been previously reported that intestinal bacteria could be utilized to transform GL directly into GA, but this transformation had to be under anaerobic environment and addition of extra nutrients was required Morana *et al*. (2002). In our study, a strain of *Trichosporon jirovecii* was selected to transform GA into GL under aerobic condition without addition of any extra nutrients.

CONCLUSION

Response Surface Methodology (RSM) was performed to optimize the process parameters for GA production from *Trichosporon jirovecii*. A significant quadratic polynomial obtained by Central Composite Design was useful for determining the optimal process parameter values of cultivation process that have significant effects on GA production. The use of GL (as inducible substrate) as carbon source is dependent on the development of economically feasible processes for GA production.

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إستخدام التصميم الإحصائي Central Composite Design للوصول إلي الظروف المثلي لإنتاج الحمض ١٨ بيتا جليسرهيتنك بإستخدام الخميرة تريكوسبورون جيروفيسياي

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الملخص العربي

في دراسة سابقة تم إستخدام تصميم Plackett-Burman لتحديد أفضل العوامل المؤثرة في إنتاج حمض الجليسرهيتينك من الجليسرهيزين المستخلص من جذور نبات العرق سوس بإستخدام الخميرة تريكوسبورون جيروفيسياي، أما في هذة الدراسة تم إستخدام تصميم Central Composed لتحديد العوامل المثلي للإنتاج. المتغيرات المستقلة المستخدمة هي درجة الحموضة، فترة التحضين، تركيز الجلوكوز، تركيز مستخلص الخميرة و مستخلص الذرة. ووجد أن التصميم Central Composed قادرعلي التنبؤ بأثر المتغيرات المستقلة علي الإستجابة. تم إستخدام تحليل التباين لتحديد مدي ملائمة التصميم المستخدم والقيمة التجريبية، وأشارت النتائج إلي أن هذا النموذج كان شبة كاف ومناسب إلي حد ما مع البيانات الفعلية وكانت قيمة r التربيعية هي (0.548). وكان أعلي تركيز متحصل علية هو ١٥٨ ملجم لكل ٠.٦ جرام من المستخلص الخام للجليسرهيزين.