Original Article

Optimization of Coenzyme Q10 Production by *Gluconobacter japonicus* FM10 Using Response Surface Methodology

Foozieh Moghadami[1]* , Ramin Hosseini[2], Jamshid Fooladi[3] , Mahdi Kalantari[4]

[1]Department of Biology, Payame Noor University, Tehran, Iran
[2]Department of Biotechnology, Faculty of Agriculture and Natural Resources, Imam Khomeini International University, Qazvin, Iran
[3]Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran
[4]Department of Statistics, Payame Noor University, Tehran, Iran

Corresponding Author: Foozieh Moghadami, PhD, Assistant Professor, Department of Biology, Payame Noor University, P.B:19395-4697, Tehran, Iran. Tel: +98-9127821623, Email: fouziehm@yahoo.com

Abstract
Introduction: CoQ_{10} is one of the antioxidants with a worldwide market. Nowadays the CoQ_{10} production has been considered by fermentation using microorganisms. In this study, the response surface methodology (RSM) was used to optimize culture composition for CoQ_{10} production by a previously isolated bacterium, *Gluconobacter japonicus* FM10.
Materials and Methods: A central composite design (CCD) was employed to optimize the culture composition including sorbitol, yeast extract, peptone, KH_2PO_4, and $MgSO_4$ for CoQ_{10} production. The dry cell weight (DCW) and CoQ_{10} concentration were monitored as response variables and the desirability function approach was applied to obtain the optimum level for each factor.
Results: Results showed that an average, 3 mg/L of CoQ_{10} was obtained when the optimized culture composition were employed (110 g/L of sorbitol, 25 g/L of yeast extract, 35 g/L of peptone, 0.5 g/L of KH_2PO_4, and 0.55 g/L of $MgSO_4$). In addition, the expected DCW reached 6 g/L in the presence of 90 g/L of sorbitol, 17.5 g/L of yeast extract, 35 g/L of peptone, 0 g/L of KH_2PO_4, and 1.7 g/L of $MgSO_4$.
Conclusions: The results of regression analysis revealed that the concentrations of peptone and sorbitol were the most effective factors in producing CoQ_{10} and DCW, respectively.
Keywords: Coenzyme Q_{10}, *Gluconobacter japonicus*, Optimization, Response Surface Methodology
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Introduction

Coenzyme Q, known as ubiquinone, is an obligatory cofactor in the aerobic respiratory chain that produces ATP.[1] It is formed from the conjugation of a benzoquinone ring with a hydrophobic isoprenoid chain of varying chain length, depending on the species.[2] Coenzyme Q_{10} (CoQ_{10}), 2, 3-dimethoxy-5-methyl-benzoquinone with 10 isoprenoid units as the side chain is the ubiquinone in the mitochondria of human cells.[3] CoQ_{10} participates as a membrane-bound redox-active molecule in several cellular functions such as the formation of disulfide bonds in proteins, detoxification of harmful ROS, controlling of cellular redox status and gene expression.[4] Recently, it has been used for the treatment of some diseases such as cancer, diabetes, heart disease, migraine, Alzheimer's and Parkinson's diseases.[5]

As the demand for CoQ_{10} increases, several attempts have been made to produce CoQ_{10} using microorganisms.[6] CoQ_{10} natural producer isolation is the most successful strategy in the microbial strains development for CoQ_{10} production.[2] A number of bacteria, including *Agrobacterium tumefaciens*,[7]

Rhodobacter sphaeroides,[3] *Paracoccus denitrificans*,[8] *Pseudomonas*,[9] *Sphingomonas*,[10] *Proteus*[11] and engineered *E. coli*[12,13] have been reported as CoQ_{10} producers. Some of these strains are able to produce low CoQ_{10}, thus there are some strategies for CoQ_{10} enhancement by bacteria such as mutation,[14,15] using the precursors,[16] metabolic engineering[12,13] and improving CoQ_{10} yield through growth conditions.[17]

Gluconobacter is a gram-negative bacterium belonging to the *Acetobacteraceae* family[18] which has been proven to be well adapted for industrial uses.[19] The main industrial important applications of *Gluconobacter* are the production of vitamin C, dihydroxyacetone, 6-amino-L-sorbose, shikimate and 3-dehydroshikimate.[20] These products are the results of incomplete oxidation performing by this genus.[21] It is indicated that there are several membrane-bound dehydrogenases located in the cytoplasmic membrane oxidizing sugars and sugar alcohols through one or more steps, and the CoQ_{10} is the part of the *Gluconobacter* respiratory chain.[22]

The response surface methodology (RSM) is a collection of statistical and mathematical techniques that are useful for

modeling and the analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response.[23] Optimization of several parameters by the method of one factor at a time is time consuming, therefore, RSM can be replaced particularly when the interactions among factors are important.[24]

In a previous study,[25] *Gluconobacter japonicus* FM10 was shown to be a CoQ_{10} natural producer. Here, the culture composition including sorbitol, yeast extract, peptone, KH_2PO_4 and $MgSO_4$ were optimized using RSM for increasing the dry cell weight (DCW) and optimistically, CoQ_{10} yield by *G. japonicus* FM10.

Materials and Methods
Microorganism and Media
The microorganism used in this study, *G. japonicus* FM10 was isolated and identified previously.[25] This strain was maintained on the GYC medium (glucose 50 g/L, yeast extract 10 g/L, $CaCO_3$ 30 g/L, Agar 25 g/L) for 2-3 months and in a frozen state at -70° C as stock. The seed culture contained 20 g/L sorbitol, 3 g/L yeast extract and 3 g/L peptone and the CoQ_{10} production culture contained sorbitol, yeast extract, peptone, KH_2PO_4 and $MgSO_4$ in the concentrations determined by RSM.

The concentration of sorbitol, yeast extract and peptone for RSM was obtained in a previous report.[25] All experiments were performed in 250-mL flasks containing 100 mL of medium with pH: 6.5, agitation speed of 180 rpm and incubation temperature of 30°C. Extraction of CoQ_{10} and measurement of DCW was performed after 40 h of growth.

Extraction and Measurement of CoQ_{10}

Extraction of CoQ_{10} was performed by the method described previously[13] with a few modifications. The cells in 1 mL of *G. japonicus* FM10 cultures were harvested at 9000 × g for 15 min. The pellets were washed with 1 mL of distilled water and suspended in 0.5 mL of the Cell Lytic B (Sigma-Aldrich). After 30 min incubation at 30°C and shaking well, 1 mL of hexane: 2-propanol (5:3) was added to the solution and mixed well. The upper phase was transferred into the new tube and after adding 0.5 mL of hexane and mixing vigorously, the upper phase was re-transferred into the tube. After evaporation, 0.5 mL of ethanol was added to the dried residue. Analysis of CoQ_{10} was performed by high-performance liquid chromatography (Agilent 1120, USA) with a Thermo scientist C18 column (250 mm× 4.5 mm× 5 µm) coupled to a UV detector with ethanol: methanol (70:30)

as the mobile phase at a flow rate of 1 mL/min. CoQ_{10} was detected at 275 nm.

Measurement of Dry Cell Weight
For the DCW determination, 1 mL of the cultures was centrifuged at 9000×g for 15 minutes, washed twice and dried at 60°C overnight to reach a constant weight.

Optimization of Medium Composition by RSM
In this research, we used a second-order polynomial as follows:

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum_i \sum_j b_{ij} X_i X_j \quad ,$$

Where, Y is the response, X_i and X_j are independent variables (or factors); and b_o, b_i, b_{ii}, b_{ij} are intercept, linear coefficient, quadratic coefficient, and interactive coefficient, respectively.

The initial concentration of five factors including carbon source (sorbitol), nitrogen source (yeast extract and peptone), KH_2PO_4, and $MgSO_4$ for CoQ_{10} production were optimized by RSM. In order to optimize the culture composition for CoQ_{10} production and the biomass increase, the RSM was performed using a two level fractional (half fraction) central composite design (CCD) with four replicates of central point. The experimental design consisted of 30 runs. The concentrations of sorbitol (X_1), yeast extract (X_2), peptone (X_3), KH_2PO_4 (X_4), and $MgSO_4$ (X_5) were chosen as the independent variables, and DCW (g/L) and CoQ_{10} level (mg/L) were chosen as the response variables. The Minitab statistical software (version 18.1) was used for experimental design and data analysis.[26] The ranges and levels of experimental variables are shown in Table 1. A total of 30 experiments were designed using the Minitab software and statistical analysis was performed to evaluate the analysis of variance (ANOVA). It is noteworthy that 95% level of confidence was used in this research.

Results
Regression Analysis of Experimental Data
The results of CCD and responses for CoQ_{10} and DCW levels produced by *G. japonicus* FM10 are shown in Table 2. It can be concluded that the highest amounts of CoQ_{10} (2.5 mg/L) were produced in runs 15, 27, and 30; and the highest amounts of DCW (5.3 g/L) were obtained in runs 21 and 27.

The regression models for the CoQ_{10} production and DCW as response variables were found in the second order polynomial equations as follows:

Table 1. Levels of Five Independent Variables Used for Central Composition Design

Variables	Names	Codes				
		-2	-1	0	1	2
X_1	Sorbitol (g/L)	90	100	110	120	130
X_2	Yeast extract (g/L)	15	20	25	30	35
X_3	Peptone (g/L)	15	20	25	30	35
X_4	KH_2PO_4 (g/L)	0	0.5	1	1.5	2
X_5	$MgSO_4$ (g/L)	0	0.25	0.5	0.75	1

Table 2. Central Composite Design and Responses for DCW and CoQ₁₀ Levels Produced by *Gluconobacter japonicus* FM10

Run Number	Sorbitol (g/L)	Yeast extract (g/L)	Peptone (g/L)	KH₂PO₄ (g/L)	MgSO₄ (g/L)	CoQ10 (m g/L)	DCW (g/L)
1	120	30	20	1.5	0.25	0.8	4.8
2	120	20	30	0.5	0.75	0.6	4.3
3	100	30	20	0.5	0.25	1.3	5.2
4	130	25	25	1	0.5	1.5	5.2
5	110	25	25	1	1	1.1	5.2
6	120	20	30	1.5	0.25	1.5	2.9
7	120	20	20	1.5	0.75	1.2	4.5
8	110	25	25	0	0.5	2.3	5.1
9	120	20	20	0.5	0.25	0.9	4.9
10	120	30	30	1.5	0.75	2.1	5.2
11	100	20	20	1.5	0.25	2.1	4.7
12	110	25	25	1	0	1.5	4.9
13	100	20	20	0.5	0.75	0.5	4.2
14	90	25	25	1	0.5	1.2	4.8
15	100	20	30	1.5	0.75	2.5	4.2
16	100	30	20	1.5	0.75	1.2	4
17	100	30	30	0.5	0.75	1.8	3.7
18	120	30	20	0.5	0.75	1.6	1.4
19	110	25	25	1	0.5	1.3	4.9
20	110	35	25	1	0.5	0.5	3.3
21	100	30	30	1.5	0.25	0.4	5.3
22	110	25	25	2	0.5	2.1	5
23	110	25	25	1	0.5	1.3	5.1
24	110	15	25	1	0.5	0.6	3.2
25	110	25	15	1	0.5	2	4.9
26	110	25	35	1	0.5	2.2	5.2
27	100	30	30	0.5	0.25	2.5	5.3
28	110	25	25	1	0.5	1.4	5.2
29	110	25	25	1	0.5	1.5	4.9
30	120	30	30	0.5	0.25	2.5	5.1

$$CoQ_{10} = 1.3512 + 0.1792\ X_3 - 0.1991\ X_2^2 + 0.1884\ X_3^2 + 0.2134\ X_4^2 + 0.3312\ X_1X_2 - 0.4063\ X_2X_4 + 0.1813\ X_2X_5 - 0.1938\ X_3X_4 + 0.3938\ X_4X_5 \quad (1)$$

$$DCW = 5.0396 + 0.4898\ X_1^2 - 0.4477\ X_2^2 + 0.6063\ X_1X_5 + 0.5313\ X_2X_4 - 0.4563\ X_3X_4 + 0.5813\ X_4X_5 \quad (2)$$

The adequacy of regression models was evaluated using ANOVA. The ANOVA results for the production of CoQ₁₀ and DCW based on equations (1) and (2) are shown in Table 3 and Table 4, respectively. From these ANOVA tables, it can be concluded that all model terms are significant (*P* value < 0.05). Additionally, the F value of 42.68 and *P* value <0.05 indicate that the response surface quadratic model (1) is significant. Also, the *F* value of 181.68 and *P* value <0.05 reveal that the response surface quadratic model (2) is significant as well. Table 5 and Table 6 show the test of significance for regression coefficients of equations (1) and (2), respectively. The *P* values

of these tables also confirm that all model terms are significant (*P* value < 0.05).

Optimum Range of CoQ10 and Biomass Production
The desirability values for the responses are shown in Figure 1 and Figure 2. The optimal levels of five factors are indicated in brackets in the figure of desirability function. The results of optimization showed that on average, 3 mg/L of CoQ₁₀ was obtained when the optimized culture composition were employed (110 g/L of sorbitol, 25 g/L of yeast extract, 35 g/L of peptone, 0.5 g/L of KH₂PO₄, and 0.55 g/L of MgSO₄). In addition, the expected DCW was 6 g/L in the presence of 90 g/L of sorbitol, 17.5 g/L of yeast extract, 35 g/L of peptone, 0 g/L of KH₂PO₄, and 1.7 g/L of MgSO₄. In this investigation, we optimized the response variables via determining the target values 3 mg/L and 6 g/L for CoQ₁₀ and DCW, respectively; instead of maximizing or minimizing the response variables since the results of optimization via maximizing and minimizing were undesirable.

Table 3. Regression Analysis Result Obtained by ANOVA for Production of CoQ$_{10}$

Source	DF	Adj SS	Adj MS	F Value	P Value
Model	9	12.5036	1.38929	42.68	0.000
Linear	1	0.7704	0.77042	23.67	0.000
X$_3$	1	0.7704	0.77042	23.67	0.000
Square	3	3.7301	1.24335	38.19	0.000
X$_2$* X$_2$	1	1.1100	1.11002	34.10	0.000
X$_3$* X$_3$	1	0.9938	0.99377	30.53	0.000
X$_4$* X$_4$	1	1.2750	1.27502	39.17	0.000
2-Way Interaction	5	8.0031	1.60063	49.17	0.000
X$_1$* X$_2$	1	1.7556	1.75562	53.93	0.000
X$_2$* X$_4$	1	2.6406	2.64063	81.12	0.000
X$_2$* X$_5$	1	0.5256	0.52563	16.15	0.001
X$_3$* X$_4$	1	0.6006	0.60063	18.45	0.000
X$_4$* X$_5$	1	2.4806	2.48063	76.20	0.000
Error	20	0.6511	0.03255		
Lack-of-Fit	17	0.6236	0.03668	4.00	0.140
Pure Error	3	0.0275	0.00917		
Total	29	13.1547			

Table 4. Regression Analysis Result Obtained by ANOVA for Production of DCW

Source	DF	Adj SS	Adj MS	F Value	P Value
Model	6	33.2178	5.53630	181.68	0.000
Square	2	14.0853	7.04264	231.11	0.000
X$_1$* X$_1$	1	6.8252	6.82516	223.97	0.000
X$_2$* X$_2$	1	5.7002	5.70016	187.05	0.000
2-Way Interaction	4	19.1325	4.78313	156.96	0.000
X$_1$* X$_5$	1	5.8806	5.88063	192.98	0.000
X$_2$* X$_4$	1	4.5156	4.51562	148.18	0.000
X$_3$* X$_4$	1	3.3306	3.33063	109.30	0.000
X$_4$* X$_5$	1	5.4056	5.40563	177.39	0.000
Error	23	0.7009	0.03047		
Lack-of-Fit	20	0.6334	0.03167	1.41	0.443
Pure Error	3	0.0675	0.02250		
Total	29	33.9187			

Table 5. Testing of the Significance of the Regression Coefficients Associated With CoQ$_{10}$

Term	Coef	SE Coef	T-Value	P Value
Constant	1.3512	0.0623	21.70	0.000
X3	0.1792	0.0368	4.86	0.000
X2*X2	-0.1991	0.0341	-5.84	0.000
X3*X3	0.1884	0.0341	5.53	0.000
X4*X4	0.2134	0.0341	6.26	0.000
X1*X2	0.3312	0.0451	7.34	0.000
X2*X4	-0.4063	0.0451	-9.01	0.000
X2*X5	0.1813	0.0451	4.02	0.001
X3*X4	-0.1938	0.0451	-4.30	0.000
X4*X5	0.3938	0.0451	8.73	0.000

The contour plots of CoQ$_{10}$ and DCW production are presented in Figure 3 and Figure 4, respectively. These contour plots not only show the mutual interactions between factors, but also give useful information about the structure of interactions. For example, in the contour plots of CoQ$_{10}$ (Figure 3), the interaction between sorbitol and yeast extract is a form of a curvature; however, the interaction between sorbitol and KH$_2$PO$_4$ was linear.

Discussion

The RSM is one of the most common statistical methods to optimize fermentation processes, which has recently been considered by a number of researchers.[24,27,28] Optimization of CoQ$_{10}$ production by various microorganisms using the RSM statistical method has also been the subject of several studies. For instance, Bule et al, by using the RSM method, were able

Table 6. Testing of the Significance of the Regression Coefficients Associated With DCW

Term	Coef	SE Coef	T-Value	*P* Value
Constant	5.0396	0.0504	100.01	0.000
X1*X1	0.4898	0.0327	14.97	0.000
X2*X2	-0.4477	0.0327	-13.68	0.000
X1*X5	0.6063	0.0436	13.89	0.000
X2*X4	0.5313	0.0436	12.17	0.000
X3*X4	-0.4563	0.0436	-10.45	0.000
X4*X5	0.5813	0.0436	13.32	0.000

to find optimal concentrations of natural precursors effective in increasing the production of CoQ_{10} by *Pseudomonas diminuta*.[16] Production of CoQ_{10} by *Rhodotorula glutamine* was performed by optimizing the pH and soybean oil added to the culture medium, using the RSM method. The results showed that the amount of CoQ_{10} increased from 10 mg/L to 39.2 mg/L and 78.2 mg/L in batch and fed-batch culture, respectively.[29] In another report, Tian et al optimized the parameters affecting the lysis of *Agrobacterium tumefaciens* cells such as acid content, temperature and time for the extraction of CoQ_{10} using the RSM, and were able to increase extraction rates up to two times.[30] Several studies have also been performed to optimize the culture medium of *Gluconobacter* strains by RSM.[31-33] In a study conducted by Wei et al, optimization of the media for increasing *G. oxydans*

cell weight was performed using the Uniform Design (UD) method. They combined the combination of culture media, namely sorbitol, yeast extract, ammonium sulfate, KH_2PO_4 and $MgSO_4$ with 9 Uniform Design methods and concluded that the highest amount of cell weight in the flask was obtained with 70 g/L of sorbitol, 17.5 g/L of yeast extract, 1.5 g/L of ammonium sulfate, 1 g/L of KH_2PO_4 and 0.2 g/L of $MgSO_4$ and reported that the most important factor in cell mass production was KH_2PO_4.[34]

In the present study, the media were studied to find the best composition for increasing the production of CoQ_{10}. The studied parameters were five compounds, sorbitol, yeast extract, peptone, KH_2PO_4 and $MgSO_4$, which were studied at five levels by RSM. The results showed that 3 mg/L of CoQ_{10} was obtained when the culture medium consisted of 110 g/L of sorbitol, 25 g/L of yeast extract, 35 g/L of peptone, 0.5 g/L of KH_2PO_4 and 0.55 g/L of $MgSO_4$. However, the suggested optimized culture compositions for DCW increased and CoQ_{10} production were different. The effect of culture compositions on DCW and CoQ_{10} varied. According to the obtained equations, the most important factor for the production of DCW was sorbitol, while the most important factor for the production of CoQ_{10} was peptone. Sorbitol and peptone were the most effective carbon and nitrogen sources for CoQ_{10} and DCW production by the FM10 strain in comparison with other carbon and nitrogen sources.[25] It is reported that sucrose and corn steep powder were the most effective carbon source and nitrogen source in CoQ_{10} and
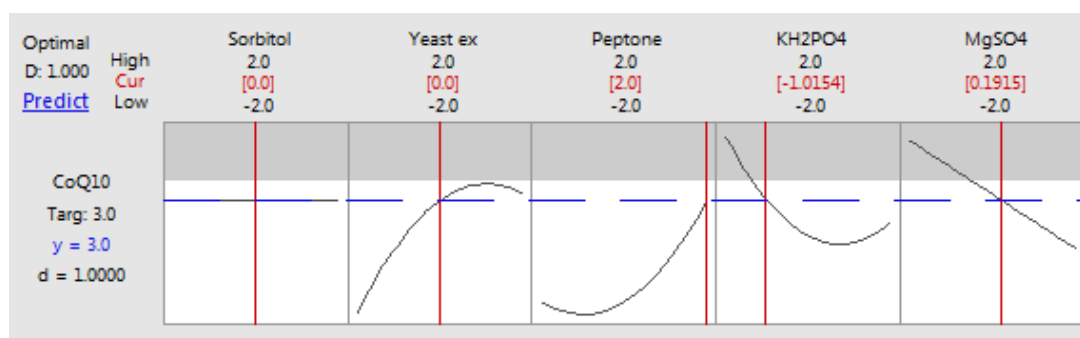
Figure 1. Target Desirability of Response for CoQ_{10} Production. The codes on the figure show 110 g/L of sorbitol, 25 g/L of yeast extract, 35 g/L of peptone, 0.5 g/L of KH_2PO_4, and 0.55 g/L of $MgSO_4$.
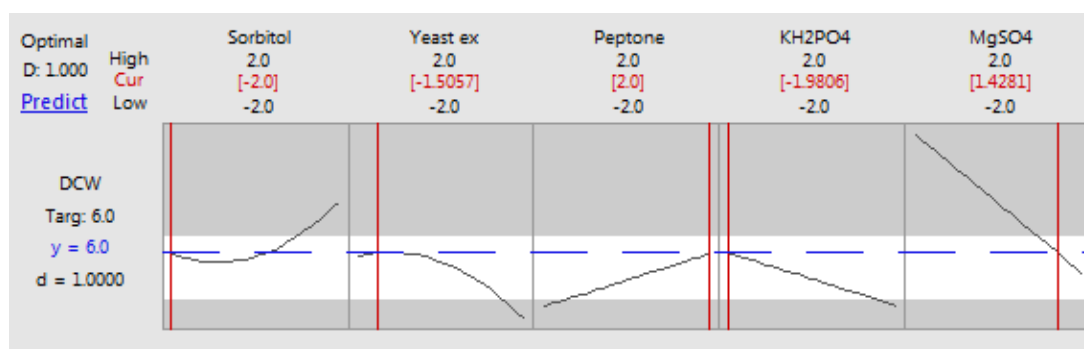
Figure 2. Target Desirability of Response for DCW. The codes on the figure show 90 g/L of sorbitol, 17.5 g/L of yeast extract, 35 g/L of peptone, 0 g/L of KH_2PO_4, and 1.7 g/L of $MgSO_4$.
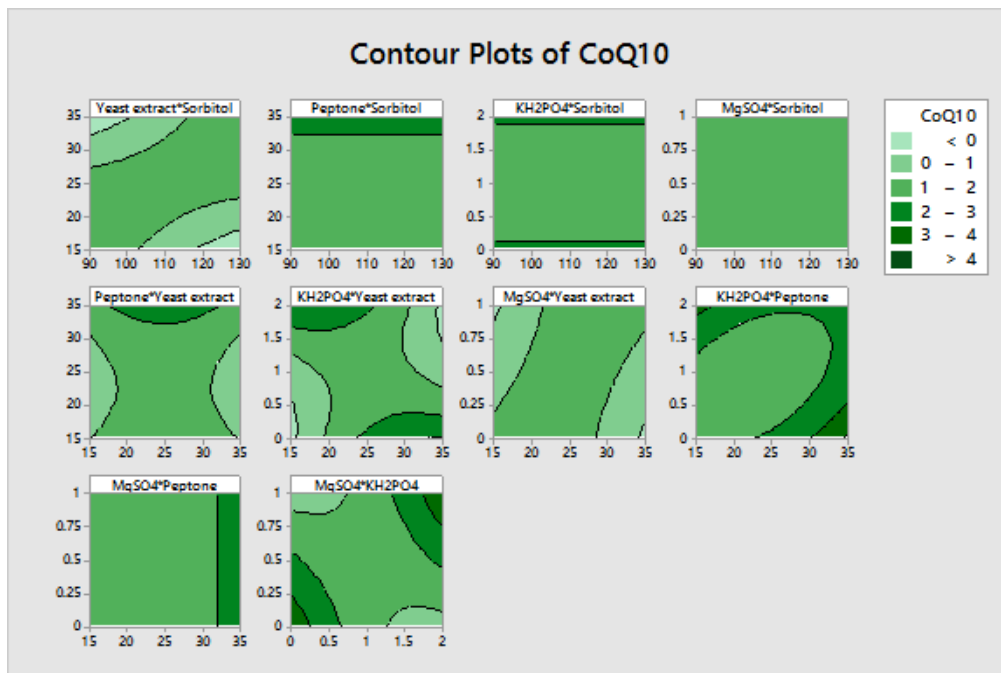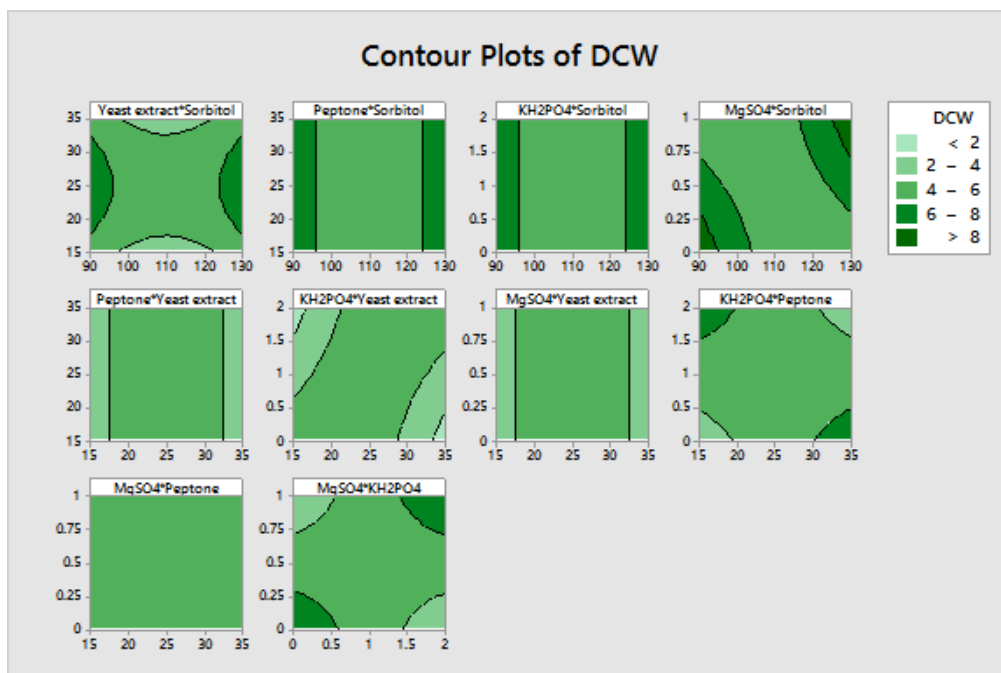
Figure 3. Contour Plots of CoQ$_{10}$.

Figure 4. Contour Plots of DCW.

DCW production in *A. tumefaciens*.[7] D-sorbitol is oxidized through membrane-bound dehydrogenases in respiratory chain of *Gluconobacter*.[22] Thus, increasing dehydrogenases activity by increasing sorbitol concentration can be effective on increasing DCW. The concentrations of culture composition in our study were slightly higher than that of Wei and colleagues'[34] research, but the amount of DCW produced by the FM10 strain (6 g/L) was significantly higher than the DCW mentioned in Wei and colleagues'[34] study (1.9 g/L).

One of the best methods used for the optimization of

multi-factorial scientific processes is the Taguchi method.[35] This approach has widespread applications in a variety of fields.[35-37] Moreover, this technique generated much debate and controversy in the statistical community about its implementation and the technical nature of data analysis. Although the Taguchi method was a valuable step towards quality improvement, it received a number of criticisms. For example, as pointed out in a previous study,[38] the Taguchi method suffers from the following drawbacks:

1– Interactions among the control factors cannot be estimated.

2– Large numbers of experimental runs are required.

3– Signal to noise ratios are unable to distinguish between inputs affecting process mean from those affecting the variance.

The RSM is a collection of mathematical and statistical tools that enable us to adopt Taguchi's robust design concept. This technique does not have the disadvantages of the Taguchi method and can provide a more statistically sound and efficient approach to analysis.

The quality of fitted models was evaluated based on the adjusted coefficient of determination denoted by R^2_{adj}. It is well known that $0 \leq R^2_{adj} \leq 1$ and values close to 1 imply that the associated regression model can be used as an appropriate predictor of response variable. The R^2_{adj} for equations (1) and (2) was 0.928 and 0.974, respectively. These values of R^2_{adj} are relatively high indicating that the proposed regression models have considerable capability to predict CoQ_{10} production and DCW with the help of five factors. The F-values of models were significant (P value <0.05), which confirmed the suitability of the proposed models. Also, the P values showed that all model terms in equations (1) and (2) were significant (P value <0.05).

Conclusions

The results of the present study indicate that using statistical techniques could enhance the yield of CoQ10 by *G. japonicus* FM10. The regression analysis revealed that the concentrations of peptone and sorbitol were the most effective factors in producing CoQ_{10} and DCW, respectively.

Author's Contributions

FM, RH and JF designed the study. FM carried out the experiments and wrote the manuscript. MK carried out the statistical analysis.

Conflict of Interest Disclosures

The authors declare no conflicts of interest.

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