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Use of carrot juice and tomato juice as natural precursors for enhanced production of ubiquinone-10 by *Pseudomonas diminuta* NCIM 2865

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ABSTRACT

Ubiquinone-10 (CoQ10) plays an essential role in the electron transport system, and has been extensively used in food and pharmaceutical industries. This paper reports on the use of statistical approach and natural precursors for enhanced production of CoQ10 using *Pseudomonas diminuta* NCIM 2865. Primarily, significance of each medium component with respect to CoQ10 production was identified by Plackett–Burman design. In the second step, concentration of most significant factors and their interaction was studied with response surface methodology (RSM). CoQ10 production increased considerably from 6.68 to 15.58 mg/l when the fermentation was carried out in the RSM optimised medium. Carrot juice and tomato juice acted as natural precursors, and enhanced the yield of CoQ10 from 15.58 to 29.22 mg/l and 24.35 mg/l, respectively.

1. Introduction

Ubiquinone-10 (Coenzyme Q10, CoQ10), a vitamin-like lipophilic component of the membrane-bound electron transport system in both prokaryotes and eukaryotes, has a highly functionalized benzoquinone moiety and an all-trans polyisoprene hydrophobic chain. It has been successfully used therapeutically for cardiovascular diseases (Khatta et al., 2000; Sarter, 2002), as supportive therapy in statin treatment (Folkers et al., 1990), and in mitochondrial respiratory-chain diseases (Geromel et al., 2002). The antioxidant activity or improved immune function of CoQ10 explains potential anti-cancer effects (Lockwood, Moesgaard, & Folkers, 1994).

The pathway for the production of carotenoids and CoQ10 is common up to geranylgeranyl diphosphate, after which it goes to the production of carotenoids directly, and to CoQ10 by addition of six molecules of IPP (Kawamukai, 2002). These reports suggest that there could be ample production of isoprenoid precursors in carrots and tomatoes. Natori and Nagasaki (1981) reported the production of active precursors of polyprenyl pyrophosphate in carrots and tomatoes.

CoQ10 can be produced by one of the three methods, namely fermentation, chemical synthesis (Negishi, Liou, Xu, & Huo, 2002) or extraction from animal tissues. Attempts have been made to produce CoQ10 using strains of bacteria, including *Agrobacterium tumefaciens* (Choi, Kim, Seo, & Ryu, 2005; Yoshida, Kotani, Ochiai, & Araki, 1998), *Paracoccus denitrificans* (Gu et al., 2006; Peter, Zgor, & Vladimir, 1993), *Rhodobacter sphaeroides* (Yen & Chiu, 2007). The wild strains of bacteria produce greater amount of CoQ10 as compared to recombinant *Escherichia coli* (Park et al., 2005; Takahashi, Nishino, & Koyama, 2003). The present work reports on the use of natural precursors for enhanced production of CoQ10 in a medium previously optimised by statistical methods. The organism used in the present study was a wild strain of *Pseudomonas diminuta* NCIM 2865.

2. Materials and methods

2.1. Materials

All the media and solvents used in the present work were of AR grade and purchased from Hi-Media Limited, Mumbai, India and S. D. Fine Chemicals Ltd, Mumbai, India. Fermented CoQ10 was a gift sample from Noreshtech Cosmeceuticals Inc, Quebec, Canada. Carrots and tomatoes were procured from a local market of Mumbai, India.

2.2. Methods

2.2.1. Organism and culture conditions

The strain *P. diminuta* NCIM 2865 was procured from National Centre for Industrial Microorganism (NCIM), Pune, India. The culture was grown on nutrient agar at 30 ± 2 °C, and maintained at 4 °C. The seed culture was grown in 250 ml Erlenmeyer flask containing 50 ml of basal medium (2% glucose, 1% peptone for bacteriology, 1% yeast extract, 0.5% sodium chloride) at 30 ± 2 °C and 180 rpm for 24 h after inoculating it with freshly grown culture.

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2.2.2. Fermentation

The fermentation was carried out in a medium reported by Natori, Nagasaki, Kobayashi, and Fukawa (1978), with slight modifications. The medium composition was 2% glucose, 1% peptone, 1% yeast extract, 0.12% $CaCO_3$ and 0.03% $MgSO_4$. The pH was adjusted to 7.2 ± 0.2 with 1 M NaOH and 1 M HCl. 10% (v/v) seed culture containing 2–4 × 10^8 cells/ml was inoculated into 50 ml of production medium. The flasks were kept on a rotary shaker (2.54 cm stroke and 180 rpm) at 30 ± 2 °C for 96 h.

2.2.3. Dry cell weight determination

Samples were diluted 50-fold with 0.02 M HCl to convert the insoluble calcium carbonate to soluble calcium chloride and carbon dioxide. The optical density of the culture broth measured at 660 nm, and the dry cell weight was estimated from calibration curve (Yoshida et al., 1998).

2.2.4. Analysis of CoQ10

To 10 ml of the bacterial culture, 12 ml of n-propanol, 20 ml of n-hexane and 10 g of glass beads were added, and the mixture was blended in a commercial blender for 5 min to extract CoQ10 from the cells. The solvent phase obtained by the second extraction from the aqueous phase with 6 ml of n-hexane were combined and evaporated to dryness on a rotavapor (Buchi Rotavapor R-124, Flawil, Switzerland) (Yoshida et al., 1998). Extracted samples of CoQ10 were dissolved in dioxane and analysed by a HPLC equipped with a Sperisorb C18 column. Elution was carried out with 100% ethanol at a flow rate of 1 ml/min (Lee, Her, Kim, & Seo, 2004). Detection was carried out with UV detector at 275 nm. The amount of CoQ10 was estimated from the calibration curve obtained with an authentic sample.

2.2.5. Glycerol estimation

Glycerol in the fermentation broth was estimated by a colourimetric procedure as described by Lynch and Yang (2004). The linear calibration curve of glycerol was obtained in the range of 0–25 μg/ml.

2.3. Optimisation of fermentation medium using statistical methods

2.3.1. Evaluation of nutritional effect by Plackett–Burman design

Screening of the most significant fermentation parameters affecting CoQ10 production was performed by Plackett–Burman design (Plackett & Burman, 1946). These factors were previously optimised using one factor-at-a-time approach (data not shown). A total of 11 (n) variables, including nine (nutritional and physical) and two dummy variables were studied in twelve ($n + 1$) experiments. Factors evaluated were like glycerol (50, 10 g/l), yeast extract (20, 5 g/l), beef extract (10, 2 g/l), ammonium dihydrogen phosphate (10, 1), calcium carbonate (10, 1 g/l), magnesium sulphate (0.5, 0.1 g/l), trace element solution (1.5, 0.5 ml/l), inoculum size (15, 5%) and $CaCO_3$ (2, 0.5 g/l) were taken from preliminary studies and additional factor like sodium chloride (10, 1 g/l) was included to study its significance on CoQ10 production. The figures in the parenthesis indicate the high and low values of the respective variables.

2.3.2. Concentration optimisation of screened component by response surface methodology (RSM)

Once the critical variables were obtained from Plackett–Burman design, RSM was performed to optimise the medium components for enhanced CoQ10 production for four independent variables. Regression analysis was performed on the data obtained from the design experiments. Experimental designs were generated and analyzed by using the statistical software package 'Design-Expert 6.0.10 Stat-Ease Inc., Minneapolis, MN, USA.

2.4. Use of carrot juice and tomato juice as natural precursors

In order to explore the effects of natural precursors on the formation CoQ10 by *P. diminuta* NCIM 2865, various concentrations of carrot juice and tomato juice were added in the culture medium. Thoroughly washed carrots were crushed in a juicer to produce quantity sufficient juice using double distilled water, and this was considered as 100% carrot juice (e.g.100 ml of juice was prepared from 100 g of carrot). This juice was filtered twice through muslin cloth. The juice so obtained was boiled for 10 min and filtered again through muslin cloth to remove all agglomerates. RSM optimised medium components were dissolved in the filtrate which acted as natural precursor for CoQ10 production. Tomato juice as a precursor was prepared similarly as carrot juice.

3. Results and discussion

3.1. Nutritional effect evaluation by Plackett–Burman design

Plackett–Burman experiments highlighted the importance of optimising culture variables in attaining higher CoQ10 production. Among the nine variables which were likely to play a significant role in enhancing CoQ10 production, four factors, namely, glycerol, yeast extract, calcium carbonate and magnesium sulphate most significantly affected CoQ10 production. None of the dummy variables incorporated in the design exhibited any impact on CoQ10 production (data not shown).

3.2. Optimization of CoQ10 production by RSM

The four significant factors that were identified by Plackett–Burman design were further optimised using RSM. Table 1 represents coded values of independent variables, design and results of CoQ10 yield, in which experiments were performed in three blocks (1–10, 11–20, 21–30). The second order regression equation provided the levels of CoQ10 production as a function of initial values of glycerol, yeast extract, $CaCO_3$ and $MgSO_4$, which could be predicted by the following equation:

$$\begin{aligned}\text{CoQ10 (mg/l)} = &-4.08 - (0.19 \times \text{Glycerol}) \\ &+ (1.39 \times \text{Yeast extract}) + (12.80 \times CaCO_3) \\ &+ (15.41 \times MgSO_4) + (3.12e^{-0.003} \times \text{Glycerol}^2) \\ &- (0.05 \times \text{Yeast extract}^2) - (2.79 \times CaCO_3) \\ &- (77.89 \times MgSO_4^2) + (8.26e^{-0.003} \times \text{Glycerol} \\ &\times \text{Yeast extract}) - (0.097 \times \text{Glycerol} \times CaCO_3) \\ &- (0.39 \times \text{Glycerol} \times MgSO_4) \\ &- (0.13 \times \text{Yeast extract} \times CaCO_3) \\ &+ (1.37 \times \text{Yeast extract} \times MgSO_4) \\ &+ (4.73 \times CaCO_3 \times MgSO_4)\end{aligned}$$

ANOVA was used to confirm the adequacy of the model, which was tested using Fisher's statistical analysis. Coefficients of estimate, sum of squares, degree of freedom, F-value and P-values are summarised in Table 2. It is clear that the linear terms of $MgSO_4$ are the least significant ($P > 0.05$). The linear terms of glycerol, yeast extract and $CaCO_3$, the squared and interactive terms of glycerol, yeast extract, $CaCO_3$ and $MgSO_4$ had high significance on correlation of coefficients with low P-values of less than 0.05. The coefficients of correlation and variation and are also given in Table 2. The mathematical model was very reliable with R^2 value of 0.9793. The low coefficient of variation (CV) value of 4.35% indicated the degree of precision with which the treatments were compared. The computed F-value of 43.98 reflected the significance of the model.

Table 1
The CCD matrix of independent variables in coded and actual form with their corresponding response.

Std. run	Glycerol (g/l)	Yeast extract (g/l)	$CaCO_3$ (g/l)	$MgSO_4$ (g/l)	CoQ10[a] (mg/l)	
					Actual	Predicted
1	30 (−1)	10 (−1)	1.2 (−1)	0.25 (1)	13.57 ± 0.92	13.44
2	50 (1)	10 (−1)	1.2 (−1)	0.1 (−1)	14.11 ± 0.98	13.74
3	30 (−1)	20 (1)	1.2 (−1)	0.1 (−1)	15.76 ± 0.14	15.50
4	50 (1)	20 (1)	1.2 (−1)	0.25 (1)	17.20 ± 0.49	17.07
5	30 (−1)	10 (−1)	2.4 (1)	0.1 (−1)	12.72 ± 0.55	12.99
6	50 (1)	10 (−1)	2.4 (1)	0.25 (1)	8.99 ± 0.05	9.38
7	30 (−1)	20 (1)	2.4 (1)	0.25 (1)	14.68 ± 0.45	15.18
8	50 (1)	20 (1)	2.4 (1)	0.1 (−1)	11.65 ± 0.52	11.91
9	40 (0)	15 (0)	1.8 (0)	0.175 (0)	16.42 ± 0.67	16.02
10	40 (0)	15 (0)	1.8 (0)	0.175 (0)	16.18 ± 0.94	16.02
11	30 (−2)	10 (−1)	1.2 (−1)	0.1 (−1)	9.74 ± 0.76	10.29
12	50 (1)	10 (−1)	1.2 (−1)	0.25 (1)	7.69 ± 0.20	8.14
13	30 (−1)	20 (1)	1.2 (−1)	0.25 (1)	13.32 ± 0.16	13.14
14	50 (1)	20 (1)	1.2 (−1)	0.1 (−1)	12.78 ± 0.49	13.03
15	30 (−1)	10 (−1)	2.4 (1)	0.25 (1)	9.85 ± 0.26	9.42
16	50 (1)	10 (−1)	2.4 (1)	0.1 (−1)	6.55 ± 0.14	6.55
17	30 (−1)	20 (1)	2.4 (1)	0.1 (−1)	9.75 ± 0.17	9.12
18	50 (1)	20 (1)	2.4 (1)	0.25 (1)	9.95 ± 0.24	9.22
19	40 (0)	15 (0)	1.8 (0)	0.175 (0)	12.04 ± 0.02	12.23
20	40 (0)	15 (0)	1.8 (0)	0.175 (0)	11.71 ± 0.18	12.23
21	20 (−2)	15 (0)	1.8 (0)	0.175(0)	16.83 ± 0.29	16.96
22	60 (2)	15 (0)	1.8 (0)	0.175 (0)	14.54 ± 0.27	14.45
23	40 (0)	5 (−2)	1.8 (0)	0.175 (0)	7.36 ± 0.31	6.97
24	40 (0)	25 (2)	1.8 (0)	0.175 (0)	11.59 ± 0.65	12.02
25	40 (0)	15 (0)	0.6 (−2)	0.175 (0)	13.14 ± 0.98	13.02
26	40 (0)	15 (0)	3.0 (2)	0.175 (0)	7.71 ± 0.33	7.88
27	40 (0)	15 (0)	1.8 (0)	0.025 (−2)	12.54 ± 0.18	12.47
28	40 (0)	15 (0)	1.8 (0)	0.325 (2)	12.83 ± 0.52	12.94
29	40 (0)	15 (0)	1.8 (0)	0.175 (0)	14.13 ± 0.79	14.46
30	40 (0)	15 (0)	1.8 (0)	0.175 (0)	14.96 ± 0.91	14.46

[a] Results are mean ± SD of three individual determinations.

Table 2
Analysis of variance (ANOVA) for the experimental results of the central-composite design (Quadratic Model).

Factor	Coefficient estimate	Sum of squares	Standard error	DF	*F*-value	Prob > *F* *P*-value
Model	14.24	177.29	0.22	14	43.98	<0.0001
A	−0.63	9.45	0.11	1	32.82	<0.0001
B	1.26	38.30	0.11	1	133.01	<0.0001
C	−1.29	39.72	0.11	1	137.94	<0.0001
D	0.12	0.32	0.11	1	1.12	0.3090
A^2	0.31	2.67	0.10	1	9.28	0.0094
B^2	−1.24	42.26	0.10	1	146.77	<0.0001
C^2	−1.00	27.58	0.10	1	95.77	<0.0001
D^2	−0.44	5.27	0.10	1	18.29	0.0009
AB	0.41	2.73	0.13	1	9.48	0.0088
AC	−0.58	5.37	0.13	1	18.66	0.0008
AD	−0.29	1.39	0.13	1	4.82	0.0469
BC	−0.38	2.28	0.13	1	7.91	0.0147
BD	0.51	4.24	0.13	1	14.72	0.0021
CD	0.21	0.73	0.13	1	2.52	0.1363

A: glycerol, B: yeast extract, C: $CaCO_3$, D: $MgSO_4$.

The optimal concentrations for the four components obtained from the model were 40, 17.72, 1.57 and 0.23 g/l for glycerol, yeast extract, $CaCO_3$ and $MgSO_4$, respectively. In order to verify the optimization results, experiments were performed under the predicted optimal conditions. The final yield of CoQ10 was 15.58 ± 0.18 mg/l, which was much closer to yield predicted by software.

3.3. Effect of carrot juice and tomato juice as natural precursors

In this study, carrot juice and tomato juice were used as natural precursors since these plants accumulate carotenoids and contain active precursors of polyprenyl pyrophosphate. The studies carried out at three different concentrations (100%, 50% and 25%) of carrot and tomato juices showed 100% carrot juice to support maximum production of 27.86 ± 0.20 mg/l of CoQ10. The yield of CoQ10 with tomato juice was lower at all respective concentrations as compared to carrot juice. The dry cell weight was higher with both precursors as compared to control. Natori and Nagasaki (1981) reported the constituents of carrot and tomato extract that strengthens CoQ10 formation to be hydrophilic in nature, is of low molecular weight. They also observed that these constituent were not extracted in organic solvents and diffused easily through dialysis membrane.

The production profile with respect to time was studied with (100%) carrot juice and tomato juice that had supported maximum CoQ10 formation. The glycerol utilization was higher with carrot juice as a precursor than tomato juice. The yield of 29.22 ± 0.51 mg/l CoQ10 and dry cell weight of 5.72 ± 0.05 g/l after 96 h was higher in carrot juice than 24.35 ± 0.012 mg/l CoQ10 and 4.9 ± 0.04 g/l biomass for tomato juice (Fig. 1). These yields are much

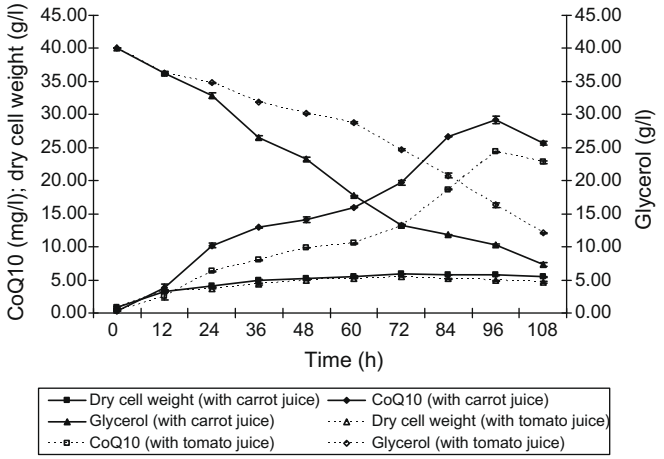

Fig. 1. Production profile for CoQ10, dry cell weight and glycerol utilization during growth of *P. diminuta* NCIM 2865 in carrot and tomato juice medium as natural precursors.

higher as compared to CoQ10 produced from wild strain of *Pseudomonas* N842 (7.47 mg/l) used by Natori and Nagasaki (1981). This could be attributed due to the presence of precursors that are common to the biosynthesis of CoQ10 and carotenoids (Kawamukai, 2002). This can be confirmed by analytical determination of such precursors, the methodology for which is not firmly established. The yield could be further increased by using various precursors like vitamins and amino acids, or by process modifications such as fed-batch fermentation. These studies are in progress.

4. Conclusions

An integrated approach of using statistical techniques and natural precursors could enhance the yield of CoQ10 by *P. diminuta* NCIM 2865. The study indicated both carrot juice and tomato juice to act as natural precursors for CoQ10 production, although the results were higher with carrot juice.

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