ANN-GA hybrid methodology based optimization study for microbial production of CoQ10

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1. Abstract

Ubiquinone-10 also known as CoQ10 is a potent antioxidant which is found at membrane-bound electron transport system and has a wide range of therapeutic use.

Purpose: The purpose of this study was to implement fermentation process optimization for production of CoQ10 by using *Pseudomonas diminuta NCIM 286*5.

Methods: Significant medium components with respect to CoQ10 production were identified using Plackett-Burman design wherein their interaction was studied using response surface methodology (RSM). CoQ10 production increased considerably from 10.8 to 18.57 mg/l when fermentation was carried out in RSM optimised medium. Further, production of CoQ10 was increased by using the predictive results of ANN-GA (artificial neural network and genetic algorithm) hybrid method.

Results and Conclusions: This increased the yield of CoQ10 18.57 to 27.9 mg/l. The experimental study using ANN-GA based optimized medium condition in the presence of carrot juice as precursor for the CoQ10 production reported as yield of 34.4 mg/l, quite high compared to the earlier studies.

Keywords: CoQ10; Process optimization; Response surface methodology; ANN-GA; Fermentation.

2. Introduction

Coenzyme Q10, also known as ubiquinone10 is a natural quinone that is found in most aerobic organisms from bacteria to mammals. It is essential component of electron transfer system, where it acts as an electron donor or acceptor between complex I/II and complex III. Ubiquinone also has vital roles in management of oxidative stress to prevent DNA damage, lipid peroxidation, protein oxidation, etc; apart from gene regulation [1, 2]. So far nine complementation groups of Q-deficient yeast mutants (CoQ1 through CoQ9) have been identified. Mammalian homologues of the yeast CoQ10 genes have been identified via sequence homology. Coq1 - Coq9 polypeptides are localized to the mitochondria. Coenzyme Q10 is a natural human quinone, found naturally in the energy producing centre of the cell, known as the mitochondria [3], but it can be chemically synthesized. Coenzyme Q10 is composed of a Ubiquinone head group (a quinone ring, capable of transferring electrons) attached to a tail of five carbon isoprenoid units. The biosynthesis of CoQ10 in humans starts with tyrosine through a cascade of eight aromatic precursors, via the mevalonate pathway. Coenzyme Q10 is commonly used for treatment of cardiomyopathy and its deficiency has been suggested to be associated with neurodegenerative diseases. In humans CoQ10 is found in relatively higher concentrations in cells with high energy requirements such as the muscle, liver, heart and pancreas.

Extensive efforts are being put to enhance the industrial CoQ10 production, using various techniques. The synthesis of CoQ10 by chemistry route was achieved by Eem and Kanan in 1988, which required harsh conditions leading to the development of a highly stereo selective process due to all E-conformations of the enzyme CoQ10 tail. Also, the process has limitations of raw materials and high cost of production of ubiquinone Q10. Research efforts are being applied on the development of potent strains by conventional mutagenesis, metabolic engineering and optimization of fermentation strategies [4]. Fermentation process using bacterium *Pseudomonas diminuta*, *Agrobacterium tumefaciens*, *Paracoccus denitrificans*, etc have been carried out for CoQ10 production [5, 6]. Practical implementations of microbial processes require controlling, monitoring and optimizing fermentation process so as to develop models that provide an accurate description of process without complexity [7,8]. The rational design of metabolic pathways in combination with engineering optimization of fermentation processes could facilitate the development of viable bioconversion processes [9, 10].In the present study, process optimization studies on CoQ10 production has been done on *Pseudomonas diminuta* based on ANN-GA method.

3. Materials and Methods

3.1 Microorganism and culture conditions

Pseudomonas diminuta NCIM 2865 was provided by National Collection of Industrial Microorganism (NCIM, Pune). It was grown on agar medium (Beef extract 3g, Peptone 5g, agar 20g, distilled water 1000 ml, pH 7.2) under agitation rate of 180 rpm at 30° C for 24 hours.

3.2 Fermentation medium and culture conditions

The fermentation process was carried out using 5% (v/v) inoculum of *Pseudomonas diminuta* seed culture. The fermentation medium was composed of Glycerol (24.32g/l), Yeast extract (9.32g/l), Beef extract (6.5 g/l), $NH_4H_2PO_4$ (13.46 g/l), $MgSO_4.7H_2O$ (0.35g/l), $CaCO_3$ (0.95g/l), L-isoleucine 1000 ppm, Thiamine hydrochloride 750 ppm and Trace element solution 1ml/l. The initial pH of the medium was adjusted to 7.2 ±0.05. Temperature was maintained at 30°C and agitation speed was controlled at 180 rpm.

3.3 Cell mass estimation: Dry cell weight method

Cell mass estimation was done by centrifugation of the fermentation broth. Pellets were then washed with distilled water by centrifugation and suspension of cells was prepared in minimum amount of distilled water. Suspension was then transferred into pre weighed aluminium cup and was put into the oven at 40°C. Samples in aluminium cups were weighed at different interval and observed it until the cell weight reached to constant.

3.4 Analysis of CoQ10

Extraction of CoQ10 was done by centrifugation of the broth to collect the cell pellet. The pellet was extracted by warming with ethanol (50 parts by volume) at 60° C for 1 hour. The above extraction was carried out thrice and the pooled extracts were diluted with water and were further extracted thrice with 1000 parts by volume of n-Hexane. The n-hexane layer was concentrated to dryness to recover 4.12 parts of yellow oil which was dissolved in 6 parts by volume of benzene and verified by thin layer chromatography [11, 12].

The quantitative estimation of the sample was done by measuring absorbance after treating with ethanol and ethyl cyanoacetate and evaluated against concentration for the standard Ubiquinone 10 [13]

3.5 Glycerol estimation

Glycerol in the fermentation broth is estimated by colorimetric procedure [14]. The linear calibration curve of glycerol was obtained in the range of 0-25 µg/ml.

3.6 Computational techniques

Designing of Plackett–Burman model was performed by using commercial software Minitab 15.0. ANN based GA study was done with the help of Matlab platform.

3.6.1 Optimization of fermentation medium using statistical methods

3.6.1.1 Evaluation of nutritional effect by Plackett-Burman design

Plackett- Burman design [15] was used to screen the most significant fermentation nutritional parameters for the CoQ10 production. These factors have been optimised separately using one factor at a time approach. Total six variables were taken for optimization. Factors like $CaCO_3$ (0.8-1.5 g/ml), $MgSO_4.7H_2O$ (0.2-0.5 g/ml), Yeast Extract (10-20 g/ml), Glycerol as carbon source (30-50 g/ml), Beef extract (3-7 g/ml) and $NH_4H_2PO_4$ (4-10 g/ml) were taken for the study.

3.6.1.2 Design of experiment with Central Composite Design (CCD)

The effect of four independent process parameters ($CaCO_3$, $MgSO_4.7H_20$, Yeast Extract and Glycerol) on CoQ_{10} production was analyzed using a Centre Composite design (CCD). It was performed at five experimental levels: -2, −1, 0, +1, +2. The range and the levels of the process parameters investigated in this study are given in Table 1. For a four-factor design, comprising of five central points, a total of 31 experiments were to be performed. The experimental levels of these variables were decided based on our preliminary experimental work and survey of literature [16]. Each experiment was performed in triplicate and the mean value reported. The numerical data obtained after performing the experiments were analyzed by the software (Minitab 15) for regression analysis and the coefficients of the regression equation generated. The results of CCD were shown in Table 2.The impact and significance of each term (linear terms, squared terms, and interaction terms) in the regression equation were tested by performing the analysis of variance (ANOVA) using Fisher's statistical analysis (data not shown). The interactive effect of the factors was analyzed with the aid of contour plots. The model regression Eq. (1) for the analysis is given below:

$$Y=\beta_o+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{44}X_4^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4+\beta_{23}X_2X_4+\beta_{24}X_2X_4+\beta_{34}X_3X_4\ldots \quad (1)$$

Where Y is the predicted response, β_1, β_2, β_3, β_4 are linear coefficients, β_{11}, β_{22}, β_{33}, β_{44} are quadratic coefficients,β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are interaction coefficients and β_0 is the constant coefficient.

3.6.1.3 Artificial neural network

An Artificial neural network is a computational program designed for nonlinear computations as well as it simulates the brain's learning process by mathematically modeling the network structure of inter connected node cells. The basic artificial neural network architecture has three layers, input layer, hidden layer and output layer. The neurons in the input and output layers are connected to the neurons of the hidden layer by adjusting weights which enable the network to compute complex associations between the input and output variables. They are used as ''black box models" of key variables whose relationship to other process entities are neither formally described nor mathematically established but are assumed to occur. To train an ANN model, a set of data containing input nodes and output nodes are fed. Back-propagation algorithm which is common in the literature has been used for generating the neural network. In this training algorithm, the error between the results of the output neurons and the actual outputs is calculated and propagated backward through the network. The algorithm adjusts the weights in each successive layer. Validation is done by presenting the network to test, a data set not used for training and then evaluating the system performance under the situation. Once the training is over, ANN is capable of predicting the output when any input similar to the pattern that it has learnt is fed. Regression based response surface models require the order of the model to be stated, while ANN tends to implicitly match the input vector (i.e., medium composition) to the output vector (Co Q_{10} production). ANN was applied here to provide a non-linear mapping between input variables concentration ($CaCO_3$, $MgSO_4.7H_20$, $NH_4H_2PO_4$, and Glycerol) and the output variable (Co Q_{10} Yield). ANN has been applied for simulating the same set of experimental data values as used for RSM. The learning rate of the network was set to values that resulted in an optimal coefficient of correlation (R^2) for the neural network.

3.6.1.4 Genetic algorithm

A genetic algorithm is based on Charles Darwin's principle of "survival of the fittest" to solve complex biological process optimization problems [17]. GA has gained popularity over traditional optimization techniques as it can solve discontinuous or non-differentiable fitness functions efficiently [18, 19, 20, 21]. In order to solve an optimization problem (regression equation), the GA randomly generates individual chromosomes which form the initial population [22, 23]. In analogy to the principle of evolution through natural selection, the chromosomes evolved in successive iterations (generations) had a better fitness value when compared to their predecessors [24].The fitness values of the progeny generated at each iteration was evaluated by the fitness function (regression equation; [22, 23]. In order to reproduce new generations, the three genetic operators namely selection, crossover, and mutation were implemented. Selection is a process in which the chromosomes with best fitness values were chosen as parents for breeding [19, 25]. Crossover is a procedure on which the GA selects a pair of parent solutions (best fitness value) in order to produce progeny, which inherits many characteristics of the parents [23, 25, 26]. The mutation is an operation, which is implemented in order to bring out diversity in the population [23]. The process continues until a near optimum solution is attained or it meets one of the termination criteria [22].

3.7 Use of carrot juice as natural precursor

Carrot juice, a potential carotene source was used as precursors for CoQ10 production in fermentation media. The preparation of carrot juice was according to the previously reported study [11]. ANN-GA optimized medium components were used in the broth in which carrot juice was present, as precursor and the production of CoQ10 was studied at various time intervals of growth of cells.

4. Results and Discussions

4.1 Carbon source optimization

Studies were carried out in triplicate in 250 ml shake flask by replacing carbon sources of the production media. Samples were withdrawn and analysed for biomass production at various intervals. The effect was of four carbon sources glycerol, glucose, fructose and sucrose on biomass production was evaluated. Fig 1 shows that glycerol is the best carbon source for Ubiquinone10 production as it showed maximum biomass production of 3.72 g/L. Glucose followed by sucrose exhibit lesser growth compared to glycerol. Earlier studies have shown that glucose gives higher production of CoQ10 than sucrose [16].

4.2 Nutritional effect evaluation by Plackett-Burman design

Plackett-Burman experiments highlighted the importance of optimizing culture variables in attaining higher CoQ10 production. Six variables were evaluated for their role in enhancing the CoQ_{10} production and it was observed that four factors namely $CaCO_3$, $MgSO_4.7H_2O$, yeast extract and glycerol were the most significant in the production of CoQ10 as shown in Fig 2.

4.2.1 Optimization of CoQ10 production through RSM

The four significant factors that were identified by Plackett-Burman design were optimised further through Response Surface Methodology as shown in. Table 2 shows the uncoded values of independent variables, experimental and RSM predicted CoQ10 yields. The second order regression equation provided the levels of

CoQ10 as a function of concentration of Glycerol, Yeast Extract, $CaCO_3$, and $MgSO_4.7H_2O$, which could be predicted through following equation

$$CoQ10\ \left(\frac{mg}{l}\right) = 18.46 - 0.6958\ (Glycerol) - 0.5923\ (CaCo3) - 0.9850\ (Yeast\ Extract) - 0.7275\ (MgSO4.7H20) - 0.9196\ (\text{Glycerol} * \text{Glycerol}) - 0.6483\ (Yeast\ Extract * Yeast\ Extract) + 0.3879(MgSO4.7H20 * MgSO4.7H20) - 0.263(Glycerol * CaCO3) + 0.8100(Glycerol * Yeast\ Extract) + 1.4688(Glycerol * MgSO4.7H20) + 0.4850(CaCO3 * Yeast\ Extract) - 0.2562(CaCO3 * MgSO4.7H20) + 1.0900(Yeast\ Extract * MgSO4.7H2O)$$

Significance of the each coefficient was done according to the corresponding T and P value. Coefficients having larger magnitude of the T value and a smaller P value can be assigned more significance. Also, P value less than 0.05 exhibits the significance of coefficient at 5% confidence level. R^2 is another parameter for evaluating the model. Finally, R^2 value for the model was 99.64%.which defines the significance of the model.

4.3 Optimization of CoQ10 production by ANN-GA based optimization method

Back propagation algorithm is a multilayer feed forward ANN with three neurons in input layer, three in the hidden layer and one in the output layer, 'Tansig' transfer function was used to model the dependence of CoQ_{10} production on independent variables such as $CaCO_3$, $MgSO_4.7H_2O$, yeast extract and glycerol. It was observed that the ANN model provided accurate predictions. The data were trained in 302 epoch with R^2 value of 0.99949 (shown in Fig 3) and mean square error value of 0.0059, shown in Fig 4.

The results depicts that ANN based training shows better correlation with the experimental CoQ_{10} level compared to that using only RSM regression model (R^2=0.99).This trained data's fitness was evaluated by fitness evaluation function using GA tool. The parameters which were taken for the GA optimization for more population size of 10, mutation rate of 0.1 and uniform cross overrate of 0.8. Results of GA based optimization are shown in Fig 5.

The optimal concentrations for the four components obtained from the model are 1.2593, 0.3415, 18.6194 and 37.3361 (g/L) for $CaCO_3$, $MgSO_4.7H_2O$, yeast extract and glycerol respectively as shown in Table 3.

In order to verify the optimization results six experiments were performed under the predicted optimal conditions. In these six experiments the observed experimental yield 27.04 mg/L was very close to the software predicted results which was 27.87 mg/L (Table 3). The optimization using ANN model linked with GA is found to be the more effective for CoQ10 production with a high degree of accuracy.

4.4 Effect of carrot juice in production of CoQ10

Carrot juice was used as natural precursors on ANN-GA optimized medium for improving the yield of CoQ10. The studies were carried out by supplementing the media with different concentrations (25%, 50%, 75%, and 100% w/v) of carrot juice. It was found that 100% carrot juice supported the maximum production of 34.4 mg/L shown in Fig. 6 which is greater than the previously reported CoQ10 yield.

4.5 Batch Production study for CoQ10 with optimized parameters

The predicted value on ANN-GA model was subjected to shake flask studies using glycerol as carbon source substitute at 180 rpm at 30^0C. The growth studies results showed maximum CoQ_{10} production approximately at 108 hrs using carrot juice as precursor was 35.0 mg/l approximate. Whereas maximum dried cell was 5.5 g/l at 108 hrs. The production is synchronous with the growth of the cell, as the carbon source level depletes in the broth at 120 hrs. The maximum specific growth rate observed is 0.080 h^{-1}, where the product yield and specific product formation rate are obtained as 0.817 x 10^{-3} (g of CoQ10/g of cells) and 6.53 x 10^{-2} h^{-1}. The obtained yield of CoQ10 is maximum and optimized by using ANN-GA model.

5. Conclusion

In the present study on optimization of CoQ10 production using *Pseudomonas diminuta* NCIM 2865, various strategies were evaluated. ANN–GA differs from conventional optimization in their ability to learn about the system without the prior knowledge of the interactions of the process variables. The data obtained from RSM was used and processed using ANN–GA for further enhancing the CoQ10 production and that (ANN–GA) was validated experimentally also. The prediction of the ANN based model was found to be superior to that of the regression based model. The batch production of CoQ10 was carried out for 120 hrs, at 200 rpm. It was observed that the growth of the cell complete at 108 hrs, as well as the CoQ10 production, which start along with the growth and extend till 140 hrs. It may be suggested that the production is exhibit mixed type fermentation. The results showed that the training of an artificial neural network with the experimental data from *Pseudomonas diminuta* NCIM 2865 fermentation was quite successful. The input space of the neural network model was optimized using GA. The ANN–GA predicted optimized enzyme activity was higher than that of predicted by regression based model. It is further concluded that the approach for ANN–GA can be varied with different data inputs and this method can be used as a viable alternative to the standard RSM

approach and also can be employed for modelling and optimization of different bioprocess systems. Finally ANN-GA optimized media with natural precursor integrated approach increased the yield of CoQ10 up to 34.4 mg/l where as carrot juice is act as precursor. The results were experimentally verified. ANN-GA optimization method is more suitable method for production of Co Q10 yield than previously reported RSM based optimization method.

6. References

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Tables and Figures:

Table1. Levels of process parameters used in the experimental design

Independent variables (g/l)	Symbols	Range				
		-2α	-α	0	+α	+2α
$CaCO_3$	X1	0.3	0.8	1.3	1.8	2.3
$MgSO_4.7H_2O$	X2	0.05	0.2	0.35	0.5	0.65
Yeast Extract	X3	5	10	15	20	25
Glycerol	X4	20	30	40	50	60

Table 2. Fermentation data used in ANN modeling

Run	**Glycerol (g/l)**	**$CaCO_3$ (g/l)**	**Yeast Extract (g/l)**	**$MgSO_4.7H_2O$ (g/l)**	**Exp. CoQ10 Yield (mg/l)**	**Predicted CoQ10 Yield (mg/l)**
1	30	0.8	20	0.2	11.67	11.74583
2	40	1.3	15	0.35	13.23	13.2
3	40	0.3	15	0.35	11.77	11.79333
4	50	0.8	20	0.5	13.1	13.26417
5	40	1.3	15	0.35	13.4	13.2
6	30	1.8	20	0.5	9.6	9.369167
7	50	1.8	10	0.2	10.74	10.8925
8	30	0.8	10	0.2	18.57	18.48583
9	40	1.3	15	0.35	13.37	13.2
10	40	1.3	15	0.05	16.3	16.20667
11	40	1.3	15	0.35	12.9	13.2
12	40	1.3	15	0.35	13.2	13.2
13	40	1.3	5	0.35	14.7	14.74167
14	30	1.8	10	0.2	17.1	16.89417
15	50	0.8	10	0.2	12.4	12.58917
16	50	1.8	10	0.5	9.8	9.6825
17	30	0.8	10	0.5	12.4	12.42583
18	30	1.8	20	0.2	11.9	12.09417
19	30	1.8	10	0.5	9.7	9.809167
20	40	2.3	15	0.35	9.4	9.42
21	60	1.3	15	0.35	8.2	8.13
22	50	1.8	20	0.5	12.4	12.4825
23	40	1.3	15	0.65	13.16	13.29667
24	30	0.8	20	0.5	10.2	10.04583
25	40	1.3	15	0.35	13.1	13.2
26	40	1.3	15	0.35	13.2	13.2
27	40	1.3	25	0.35	10.8	10.80167
28	50	1.8	20	0.2	9.4	9.3325
29	50	0.8	20	0.2	9.2	9.089167
30	50	0.8	10	0.5	12.6	12.40417
31	20	1.3	15	0.35	10.8	10.91333

Table 3. ANN-GA based optimum solutions and experimentally validated results

Experiment No.	$CaCO_3$ (g/l)	$MgSO_4.7H_2O$ (g/l)	Yeast Extract (g/l)	Glycerol (g/l)	GA predicted CoQ10 Yield (mg/l)	Experimental CoQ10 Yield (mg/l)
1	1.2593	0.3415	18.6194	37.3361	27.87	27.04
2	1.006	0.28	19.29	39.5212	27.77	22.9
3	1.18	0.24	18.01	34.52	27.9	24.3
4	0.825	0.205	19.41	42.4463	26.82	22.8
5	1.2772	0.2679	18.1548	34.6792	28.22	23.7
6	1.1429	0.4173	19.6865	41.1081	25.09	21.3

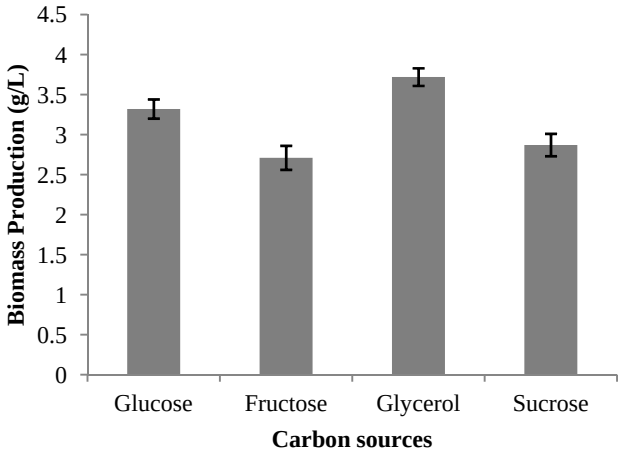

Fig.1. Effect of different carbon sources on biomass production

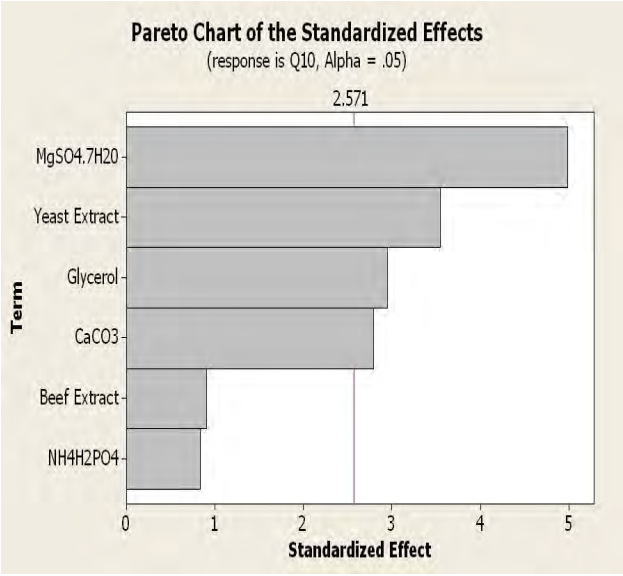

Fig.2. Pareto Chart showing the significance of four factors

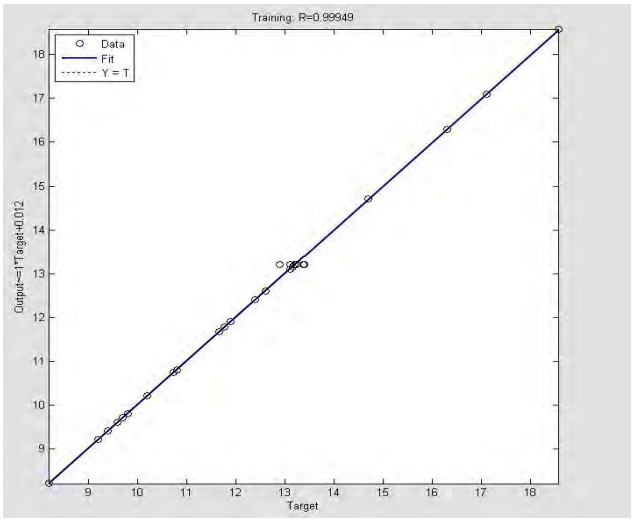

Fig.3. Regression plot of ANN trained data

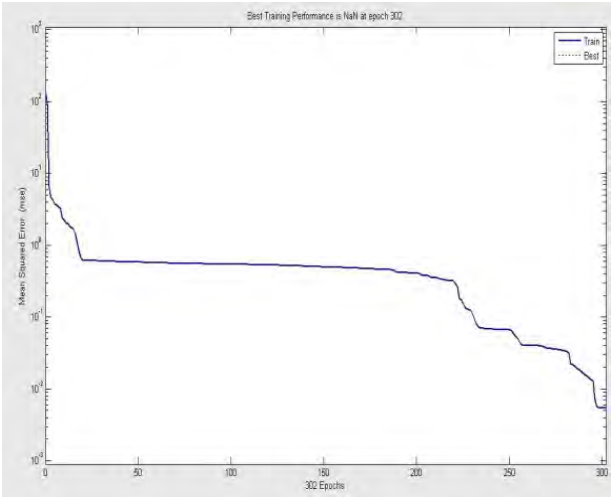

Fig.4. Figure showing the mean square error of ANN trained data

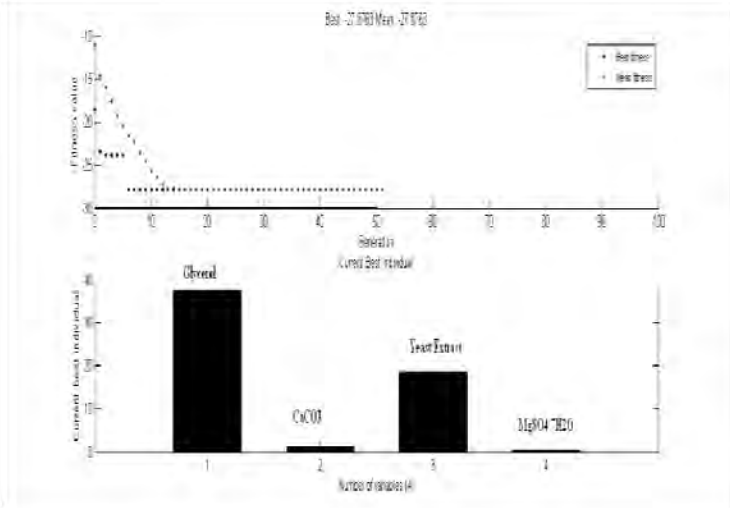

Fig.5. Fitness plot depicting the performance of GA

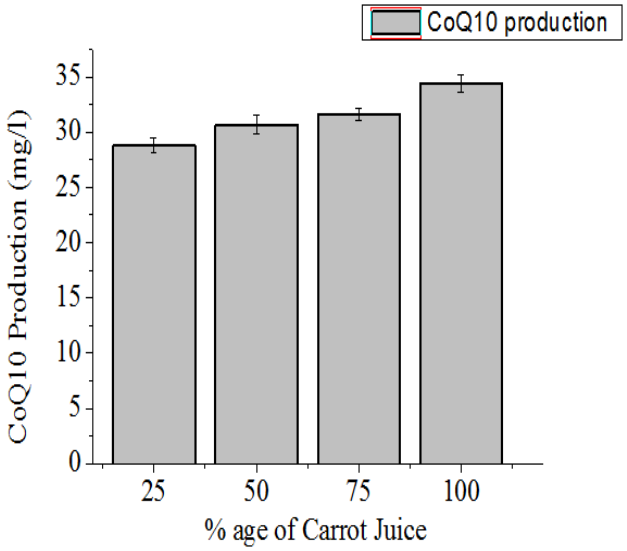

Fig.6. Variation in CoQ10 production with varying percentage (w/v) of carrot juice supplementation

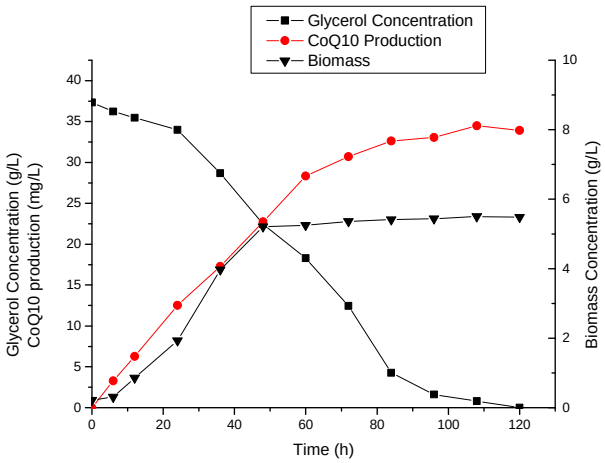

Fig.7. Production profile of Q10 and utilization of glycerol in presence of carrot juice