

Optimization of Fermentation Process Conditions for the Production of CoQ₁₀ using *Paracoccus denitrificans* ATCC 19367 Fusant Strain PF-P1

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Abstract—The process for the production of Coenzyme Q₁₀ (CoQ₁₀) from a novel fusant strain PF-P1 has been scaled up to 2 L laboratory fermenter level. At laboratory fermenter level, the strain PF-P1 produced 113.68 mg/L of CoQ₁₀ at 96 h using fed batch [intermittent dosing of para Hydroxy Benzoic Acid (pHBA) at a concentration of 25 mg/L (final concentration) at 24 h followed by 30% of sucrose solution at 48 h and 72 h respectively] fermentation with optimized production medium containing carbon source as cane molasses and showed specific CoQ₁₀ content of 2.4448 mg/g of dry cell weight (DCW). Optimized process resulted in significant improvement in biomass, CoQ₁₀ titer and specific CoQ₁₀ content than shake flask fermentation with PF-P1 strain.

Keywords— Fusant PF-P1; Specific CoQ₁₀ Content; Process Optimization.

I. INTRODUCTION

Recently CoQ₁₀ has received great attention for its application as therapeutic agent as well as in related fields such as a potential antioxidant [1]. CoQ₁₀ can be produced by chemical synthesis [2], semi-chemical synthesis [3], extraction from animal tissues [4] and microbial fermentation [5] including bacteria (e.g. *Agrobacterium*, *Paracoccus*, *Cryptococcus*, *Rhodobacter*, *Tricosporon*), molds (e.g. *Neurospora*, *Aspergillus*), yeasts (e.g. *Candida*, *Sporidobolus*, *Rhodotorula*), etc. In the wake of environmental awareness, the first three options became least desirable due to inherent uses of solvents and chemicals in the process. Microbial fermentation, on the contrary, offers an environmentally benign option based on the enzymatic catalysis at the cellular level for CoQ₁₀ assembly. Moreover, this approach is attractive to the industry because the process is easy to control at a relatively low production cost. But the potency and the capability of the producing strain are the key factors for an economically viable biological process. Due to the low levels of CoQ₁₀ in microorganisms, intensified efforts in the development of bioprocesses have been made for the economic production of CoQ₁₀ [6]. Also due to the limited genetic tools available for the strains that have been improved by mutagenesis, the transfer of engineering strategies to *E.coli*

becomes unfeasible. As a result, the research has been mainly focused on increasing CoQ₁₀ production by fine-tuning the growth conditions of these organisms, both in flask and fermenter [7]. The different strategies for isolation and purification of CoQ₁₀ from the fermentation broth have been reported [8]. The coupled fermentation-extraction process and two-phase conversion system has been reported with enhanced production of CoQ₁₀ by *Sphingomonas* sp. [9, 10]. The fermentation optimization process for *A. tumefaciens* have been reported with respect to restricted electron flux, viscosity of the broth, controlling sucrose concentration and NADH/NAD⁺ ratio [11-15]. Researchers also showed improved process for production of CoQ₁₀ from *S. johnsonii* by feeding pHBA precursors [16]. They reported the method of isolation of CoQ₁₀ based on normal phase chromatography which was useful in separating the closely eluting impurity-triacylglycerol.

In the present study, the fermentation process optimization studies for the production of CoQ₁₀ have been performed using a novel fusant strain PF-P1.

II. MATERIALS AND METHODS

A. Strain, Media, Materials and Instruments

The fusant strain PF-P1 was maintained at 4°C on Tryptic Soya Agar (TSA) slants [17]. The commercial grade raw materials and media components were procured from Hi-Media, India. All AR grade solvents used were procured from Merck. For HPLC purpose, HPLC grade solvents (Merck) were used. Absolute alcohol was purchased from Jiangsu Huaxi International Trade Co. Ltd, China. Antifoam (Desmophen) for the process was procured from Bayer's, Germany. Sorvall RC 5C centrifuge was used for separating biomass. The rotary evaporator from Buchi, Germany was used for concentration of solvents. Eutech make pH meter calibrated with standard buffers was used to measure pH. Agilent make HPLC system with PDA Detector was used for analytical HPLC.

B. Shake Flask Fermentation

The seed was prepared from 48 h growth of the culture on 50 ml MSM-3 medium (consisting of sucrose 60 g/L, peptone 15 g/L, yeast extract 15 g/L, NaCl 5 g/L, pH 7.2) in 500 ml flask, inoculated with PF-P1 from TSA slants. The 200 ml of PM-D medium (consisting of cane molasses 80 g/L, (NH₄)₂SO₄ 13 g/L, K₂HPO₄ 0.5 g/L, KH₂PO₄ 0.5 g/L, MgSO₄

7H₂O 0.25 g/L, CaCO₃ 20 g/L, Corn Steep Liquor 40 g/L, pH 7.2±0.05) dispensed in 1000 ml flask was inoculated with 10% seed and incubated on rotary shaker at 30°C with shaking at 220 rpm. The fermentation kinetics was observed till 144 h. The intermittent dosing was carried out with combination of 25 mg/L pHBA (final concentration) at 24 h followed by 30% of sucrose solution at 48 h and 72 h respectively [18]. The samples were aseptically withdrawn at different time interval from each flask for analysis of pH, DCW (g/L), CoQ₁₀ production (mg/L) and residual sugar (g/L). For each set of withdraw at different time points, three sets of conical flasks were used and mean value was only considered for the study.

C. Process Optimization in Fermenter

Fermentation process optimization studies were carried out in Applikon fermenter from Applikon Biotechnology (V1.60, ez-Control, Make-Netherlands) 2 L capacity glass fermenter. The seed inoculum was prepared in two 500 ml Erlenmeyer's flasks containing 50 ml of MSM-3 medium (consisting of sucrose 60 g/L, peptone 15 g/L, yeast extract 15 g/L, NaCl 5 g/L, pH 7.2±0.05). The flasks were inoculated with PF-P1 from TSA slants and incubated on rotary shaker at 28-30°C and 220 rpm for 48 h. A 10% (v/v) seed culture was inoculated into a 2 L fermenter with a working volume of 1 L. The PM-D medium (consisting of cane molasses 80 g/L, (NH₄)₂SO₄ 13 g/L, K₂HPO₄ 0.5 g/L, KH₂PO₄ 0.5 g/L, MgSO₄ 7H₂O 0.25 g/L, CaCO₃ 20 g/L, Corn Steep Liquor 40 g/L, pH 7.2±0.05) was used for fermenter studies. The 0.04% of Desmophen was added during the process as antifoam agent. The fermentation process optimization was carried out by one variable at a time by altering the parameters i.e. temperature (26°C, 29°C, 32°C, 35°C), aeration (0.1 vvm, 0.3 vvm, 0.5 vvm, 0.8 vvm), agitation (300 rpm, 500 rpm, 700 rpm, 900 rpm), DO (10%, 20%, 30% and 40%) of appropriate air saturation and autocontrol pH (6.0±0.05, 6.5±0.05, 7.0±0.05, 7.5±0.05) using 10% NaOH – 10% ortho phosphoric acid (OPA). The combination of intermittent dosing was carried out with 25 mg/L pHBA (final concentration) at 24 h followed by 30% of sucrose solution at 48 h and 72 h respectively [18]. The initial fermenter kinetics for pilot batch (using process parameters e.g. 29°C, 500rpm, 0.5 vvm) was run till 120 h to observe the kinetics during fermentation in comparison with shake flask study. During process optimization studies the fermenter was run 108 h. The broth samples were analyzed for pH, DCW (g/L), CoQ₁₀ production (mg/L) and residual sugar (g/L). Three sets of trials performed to optimize each set of parameters and mean value only considered for process optimization study.

D. Estimation of Total Residual Sugar

A Standard stock solution was prepared by dissolving 100 mg of sucrose in 100 ml of distilled water. The Anthrone reagent was freshly prepared by dissolving 200 mg of Anthrone reagent in 100 ml of ice cold 95% H₂SO₄. The standard curve was prepared by taking 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the standard stock and the volume was made up to 1 ml by adding distilled water. 4 ml of Anthrone reagent was added and the mixture was heated for 10 min in a boiling water bath. After rapid cooling, the absorbance was recorded at a wave length of 630 nm using SPECTRONIC® GENESYS

TM5 Spectrophotometer [19]. The fermentation broth (shake flask and/or fermenter) samples withdrawn at different time intervals were analyzed for total residual sugar content using Anthrone method. Culture supernatant obtained after centrifugation was subjected to total residual sugar estimation. 1 ml of culture supernatant was taken and diluted for 100 times (1:100 dilution) using distilled water. The 0.2% of Anthrone reagent was freshly prepared in 95% H₂SO₄. 1ml (after 1:100 dilutions) of the above diluted culture supernatant sample was taken in test tube and 4 ml of freshly prepared Anthrone reagent was added to the tube. The mixture was heated for 10 min in a boiling water bath. After rapid cooling, the absorbance was recorded at a wave length of 630 nm using SPECTRONIC® GENESYS TM5 Spectrophotometer. The amount of total residual sugar in each sample was determined by using the standard calibration curve.

E. Measurement of Dry Cell Weight (DCW)

The 10 ml of whole broth was treated with 0.002 (N) (1:100 dilutions) HCl and then centrifuged at 12000 rpm for 20 min in a pre-weighed centrifuge tube (Make: Sorvall 5C Centrifuge). The dilution was made in 0.002 (N) HCl in order to dissolve the insoluble CaCO₃ of the media, which would interfere in the reading. The cell mass was quantified by drying at 60°C until a constant mass was obtained. The dried biomass was weighed to obtain a dry cell weight (DCW) and expressed in g/L.

F. Extraction and Quantification of CoQ₁₀

The 20 ml of culture broth was centrifuged at 12000 rpm for 20 min to get biomass pellet, which was extracted with 20 ml of ethanol by heating in shaking water bath at 60°C for 3 h. The cells were removed by centrifugation and ethanol layer was re-extracted with 20 ml of hexane. The hexane layer was separated, concentrated till dryness and finally reconstituted with 1 ml of mobile phase and 20 µl was injected for HPLC analysis. The HPLC (Agilent 1100) analysis was performed by using reverse phase Unisphere C-18 column (150 mm x 4.6 mm, 3 µM particle size) and acetonitrile: isopropyl alcohol (70:30) as mobile phase with a flow rate of 1 ml/min. Detection was carried out at 275 nm. The titre was estimated by comparing the area of sample and standard CoQ₁₀ (Sigma, C9538) of known concentration (1.02 mg/ml) and expressed as mg of CoQ₁₀ per litre of broth (mg/L). The titre value was divided with DCW (g/L) to get specific CoQ₁₀ content (mg/g of DCW).

G. Statistical Analysis

For analysing differences between two groups, Student's t-test was used based on PRISM-5 software. p values below 0.05 were considered statistically significant. The values in all graphs are an average of three trials. All error bars represent standard error of mean.

III. RESULTS AND DISCUSSIONS

A. Kinetics of Shake Flask Fermentation

Before undertaking fermenter process optimization studies for strain PF-P1 using optimized fed batch strategy [18], the time course of shake flask fermentation was carried out to understand the progress of fed batch fermentation in

shake flask. Fig. 1 (a) described the titre, specific CoQ₁₀ content and DCW obtained during shake flask fermentation. The rise in DCW was gradual till 96 h and afterwards there was a slow rise till 120 h. The maximum DCW of 24.8 g/L was obtained at 120 h, afterwards it became stagnant resulted in the stagnation of CoQ₁₀ content because it attained the stationary phase. The CoQ₁₀ titre and specific CoQ₁₀ content was gradually found to increase with DCW indicating that the CoQ₁₀ production is growth associated. It can be seen from time course that combination of intermittent dosing of 25 mg/L of pHBA (final concentration) at 24 h followed by 30% of sucrose solution at 48 h and 72 h respectively helped in maintaining the exponential growth phase till late hours thereby improving CoQ₁₀ titre. The maximum CoQ₁₀ production of 37.5 mg/L was achieved at 120 h which corresponds to the specific content of 1.51 mg/g of DCW. Fig. 1 (b) described the residual sugar and pH trend obtained during shake flask fermentation. The pH was found to incline during initial 24 h and then decline rapidly till 60 h. After 60 h, one more pH drop has been noticed due to dosing of 30% sucrose at 72 h. After 72 h, it increased gradually till 144 h and reached around pH 7.04. This dosing of 30% sucrose solution intermittently at 48 h, 72 h respectively helped in maintaining the sugar concentration above 7-10 g/L till late of 96 h of fermentation cycle resulted in the biomass build-up.

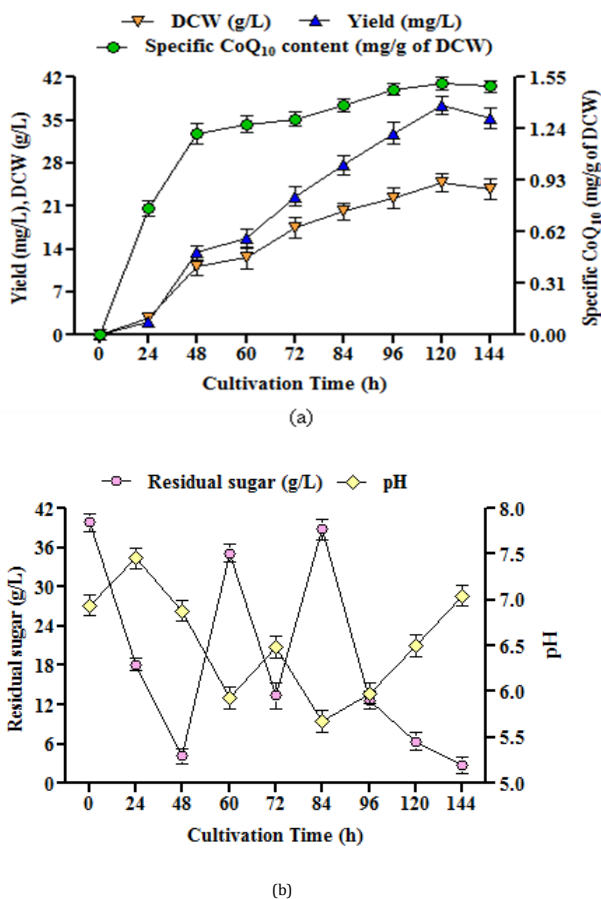


Fig. 1 Time course of fed batch fermentation for PF-P1 at shake flask (a) Kinetics for titre, DCW and specific CoQ₁₀ content during cultivation, (b) kinetics for residual sugar and pH during cultivation (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

B. Process Optimization Studies in 2 L Fermenter

The optimized fed batch process from the shake flask was transferred to the 2 L fermenter level with additional optimization studies with respect to fermenter parameters. For this purpose a superior novel fusant strain PF-P1 was used [17]. In order to make the fermentation process cost effective, the superior fusant PF-P1 and cheaper raw materials were utilized during process optimization. During fermentation of *P. denitrificans* ATCC 19367 on cane molasses based PM-D medium at shake flask level, intermittent dosing was carried out using combination of 25 mg/L of pHBA (final concentration) at 24 h followed by 30% of sucrose solution at 48 h and 72 h respectively [18]. The sugar concentration was maintained at above 7-10 g/L till late 96 h with the help of intermittent dosing of 30% sucrose at 48 h and 72 h respectively. The same fed batch strategy was adopted for further optimization in fermenter by altering the parameters using fusant PF-P1. Initially, a pilot batch was carried out using following moderate process parameters; temperature: 29°C, aeration: 0.5 vvm, agitation: 500 rpm. The time course of pilot batch is shown in Fig. 2. During fed batch fermentation in shake flask, the maximum DCW and CoQ₁₀ production were observed at 120 h of fermentation cycle may be due to limitation in mass transfer during fermentation whereas, in the fermenter pilot run it was observed that the maximum production was achieved at 96 h, after that there was a stagnation which may be due to initiation of the stationary phase. So the subsequent batches during fermenter process optimization studies were carried out till 108 h. A marginal improvement in biomass and CoQ₁₀ titre was observed may be due to better utilization of substrates during fermentation. At 96 h, 40.9 mg/L CoQ₁₀ titre was obtained with DCW of 26.1 g/L.

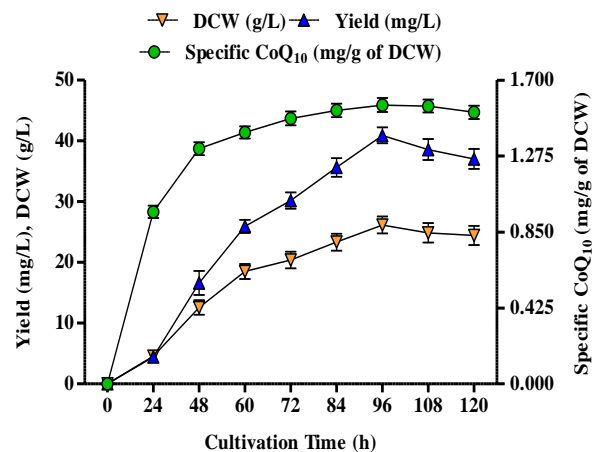


Fig. 2 Time course of fed batch fermentation for PF-P1 using moderate process parameters (temperature: 29°C, aeration: 0.5 vvm, agitation: 500 rpm). Kinetics for titre, DCW and specific CoQ₁₀ content during cultivation (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

Since CoQ₁₀ is an intracellular product, the primary aim was increasing specific CoQ₁₀ content (mg/g of DCW) rather than titre (mg/L). It was well observed in Figure 2 that 70-80% of production of CoQ₁₀ with respect to titre (mg/L)

observed during logarithmic growth phase and the specific CoQ₁₀ content (mg/g of DCW) was also rising. As the growth proceeds and approaches towards 96 h of growth phase, there was maximum production and DCW, resulting in higher specific CoQ₁₀ content than that observed during early logarithmic phase. After 96 h, as the DCW became stagnant, resulted in the reduction of specific CoQ₁₀ content. In order to observe the full growth profile the initial pilot batch with moderate process parameters was continued till 120 h and later on the fermentation cycle was reduced to 108 h. However, no significant improvement in specific CoQ₁₀ content was observed as compared to fed batch fermentation in shake flask. The residual sugar and pH trend were almost similar as it obtained with strain PF-P1 during fed batch fermentation using combination of intermittent dosing of 25 mg/L of pHBA (final concentration) at 24 h followed by 30% of sucrose solution at 48 h, 72 h respectively at shake flask level. The DO profile was not monitored during the initial optimization studies with respect to temperature, aeration and agitation. Initially DO was kept 100% (before seed inoculation) and it was uncontrolled during the process.

C. Optimization of Temperature in Fermenter

During optimization of temperature, fermenter batches were taken with variable temperatures like 26°C, 32°C, 35°C along with the initial pilot run which was taken at 29°C with constant parameters like agitation (500rpm) and aeration (0.5vvm). Temperature was found to be one of the crucial parameter for optimization using fusant PF-P1 in 2 L fermenter. It was observed that lowering the temperature from 29°C to 26°C during the fermentation resulted in a decrease in DCW as well as CoQ₁₀ production; hence 26°C may not be suitable for optimum growth and efficient utilization of substrates. Increase in temperature from 29°C to 32°C showed gradual increase in DCW and CoQ₁₀ production but beyond that temperature, there was no improvement in DCW as well as CoQ₁₀ titre. Decrease in DCW as well CoQ₁₀ titre resulted in decrease of specific CoQ₁₀ content at 26°C where as there was increase in specific CoQ₁₀ content obtained at 32°C. The optimum temperature for CoQ₁₀ production in 2L fermenter was found to be 32°C. The DCW, CoQ₁₀ titre and specific CoQ₁₀ content obtained with different temperature is shown in Table 1.

TABLE 1. Comparison of results at different temperature in fermenter for strain PF-P1 (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

Agitation (rpm)	Aeration (vvm)	Temperature (°C)	Fermentation Cycle (h)	DCW (g/L)	Titer (mg/L)	Specific CoQ ₁₀ Content (mg/g of DCW)
500	0.5	29	108	26.1	40.9	1.5605
500	0.5	26	108	17.1	25.42	1.4903
500	0.5	32	108	28	45.48	1.6288
500	0.5	35	108	22.5	33.66	1.4979

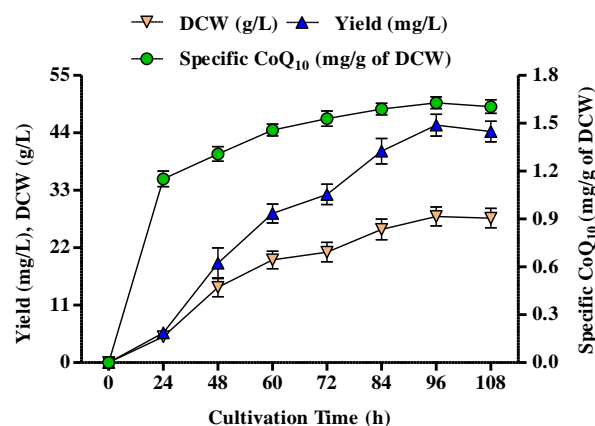


Fig. 3 Time course of fed batch fermentation for strain PF-P1 at optimized temperature condition (parameters like temperature: 32°C, aeration: 0.5vvm, agitation: 500rpm). Kinetics for titre, DCW and specific CoQ₁₀ content during cultivation (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

The time course of the fermenter batch run at optimized temperature (32°C) is shown in Fig. 3. At 32°C, the highest titre of 45.48 mg/L was observed at 96 h with 28 g/L of DCW and 1.6288 mg/g of DCW, while keeping aeration 0.5 vvm and agitation 500 rpm. The residual sugar and pH trend were found to be similar to that observed with pilot fermenter batch using moderate parameters. Initially DO was kept 100% (before seed inoculation) and it was uncontrolled during the process.

Both agitation and aeration are involved to a different extent in overall mass and oxygen transfer in the process fluid. Agitation controls the nutrient transfer and distribution of air and oxygen. Aeration not only determines the oxygenation of the culture, but also contributes to bulk mixing of the fermentation broth. High agitation promotes good mass transfer but is energy-intensive which increases the production cost. Agitation creates shear forces which cause morphological changes, variation in their growth and product formation, and also damages the cell structure. Hence optimization of aeration and agitation is essential to maintain suitable growth conditions for improved production of CoQ₁₀.

D. Optimization of Aeration in Fermenter

The fermentation process conditions were optimized for CoQ₁₀ production in 150-L reactor using a mutant strain of *R. sphaeroides* with respect to temperature, aeration and fed batch strategy [6]. They have observed that, aeration shift from the adequate aeration at early growth phase to the limited aeration in active cellular metabolism, was a key factor in CoQ₁₀ production. The combined effect of aeration/agitation and fed batch strategy on CoQ₁₀ production by *Pseudomonas diminuta* was reported [20]. The effect of limited supply of air on CoQ₁₀ production by *R. sphaeroides* was studied. The high aeration decreased the CoQ₁₀ content [5]. Earlier it was described that high aeration suppresses the CoQ₁₀ production in *Paracoccus denitrificans* [21]. During optimization of aeration condition in fermenter, trials were performed using different aerations like 0.1 vvm, 0.3 vvm, 0.5 vvm, 0.8 vvm respectively while keeping agitation (500rpm) and temperature (32°C) constant. Lowering the aeration from 0.5

vvm to 0.3 vvm during the fermentation resulted in a increase in DCW as well as CoQ₁₀ titre, but further decrease in aeration like 0.3 vvm to 0.1 vvm was not suitable for biomass build up as well as CoQ₁₀ production. It was also observed that rise in aeration from 0.5 vvm to 0.7 vvm showed gradual decrease in DCW and CoQ₁₀ titre. Hence the aeration of 0.3 vvm was found to be suitable for optimum growth and efficient utilization of substrates. The rise in DCW and COQ₁₀ titre resulted in increasing the specific CoQ₁₀ content. It has been observed that, the maximum specific CoQ₁₀ content was obtained with 0.3 vvm of aeration. The DCW, CoQ₁₀ titre and specific CoQ₁₀ content obtained with different aeration is shown in Table 2.

The time course of the batch run at optimized aeration (0.3 vvm) is shown in Fig. 4. At 0.3 vvm, the highest titre of 54.21 mg/L was observed at 96 h with 32.3 g/L of DCW and 1.6821 mg/g of DCW keeping temperature 32°C and agitation 500 rpm. The residual sugar and pH trend were found to be similar to that observed with pilot fermenter batch using moderate parameters as well as observed during optimized temperature condition when operated with the parameters like temperature 32°C, aeration 0.5 vvm and agitation 500 rpm. Initially DO was kept 100% (before seed inoculation) and it was uncontrolled during the process.

TABLE 2. Comparison of results at different aeration in fermenter for strain PF-P1 (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

Agitation (rpm)	Aeration (vvm)	Temperature (°C)	Fermentation Cycle (h)	DCW (g/L)	Titer (mg/L)	Specific CoQ ₁₀ Content (mg/g of DCW)
500	0.1	32	108	26.2	39.33	1.5013
500	0.3	32	108	32.3	54.21	1.6821
500	0.5	32	108	28	45.48	1.6288
500	0.8	32	108	28.1	43.76	1.5576

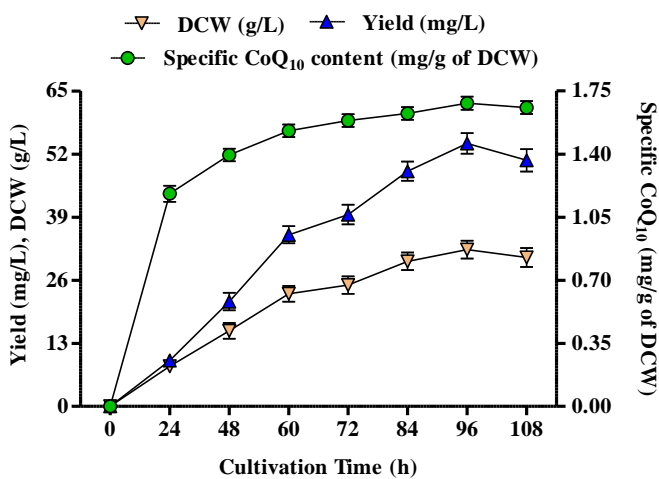


Fig. 4 Time course of fed batch fermentation for PF-P1 at optimized aeration condition (parameters like temperature: 32°C, aeration: 0.3 vvm, agitation: 500 rpm). Kinetics for titre, DCW and specific CoQ₁₀ content during cultivation (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

E. Optimization of Agitation in Fermenter

During optimization of agitation condition in fermenter, trials were performed using different agitations like 300 rpm, 500 rpm, 700 rpm and 900 rpm respectively while keeping aeration (0.3 vvm) and temperature (32°C) constant. It was observed that rise in agitation from 500 rpm to 700 rpm showed improvements in DCW and CoQ₁₀ titre but beyond 500 rpm it was gradually decreased due to high shear stress. Lowering the agitation from 500 rpm to 300 rpm showed reduction in DCW as well as CoQ₁₀ titre. The improvement in maximum specific CoQ₁₀ content was obtained at 700 rpm as compared to other agitation. The DCW, CoQ₁₀ titre and specific CoQ₁₀ content obtained with different agitation is shown in Table 3.

The time course of the batch run at optimized agitation (700 rpm) is shown in Fig. 5. At 700 rpm, the highest titre of 62.7 mg/L was observed at 96 h with 35.6 g/L of DCW and 1.7622 mg/g of DCW keeping temperature 32°C and aeration 0.3 vvm. The residual sugar and pH profile were found to be similar to that observed with pilot fermenter batch using moderate parameters as well as observed in the fermenter batches with the optimized temperature as well as aeration conditions. Initially DO was kept 100% (before seed inoculation) and it was uncontrolled during the process.

TABLE 3. Comparison of results at different agitation in fermenter for strain PF-P1 (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

Agitation (rpm)	Aeration (vvm)	Temperature (°C)	Fermentation Cycle (h)	DCW (g/L)	Titer (mg/L)	Specific CoQ ₁₀ Content (mg/g of DCW)
300	0.3	32	108	26.4	39.89	1.5166
500	0.3	32	108	32.3	54.21	1.6821
700	0.3	32	108	35.6	62.7	1.7622
900	0.3	32	108	28	41.53	1.4862

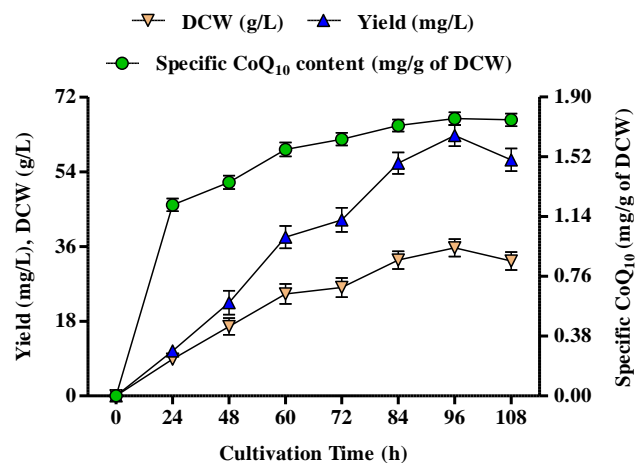


Fig. 5 Time course of fed batch fermentation for PF-P1 at optimized agitation condition (parameters like temperature: 32°C, aeration: 0.3 vvm, agitation: 700 rpm). Kinetics for titre, DCW and specific CoQ₁₀ content during cultivation (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

The generalized DO trend along with residual sugar profile observed during optimization of parameters with respect to temperature, aeration and agitation is shown in Fig. 6. During initial 6 h, due to rapid utilization of substrates leading to rapid biomass build up, DO levels dropped drastically. After 30 h, it started rising due to depletion of sugar concentration in the medium and reaches around 35% at 48 h. Due to intermittent dosing of 30% of sucrose solution at 48 h, it has been observed that the DO profile again drops down and reaches less than 5% at 60 h of fermentation cycle. Due to availability of sufficient sugar concentration in the medium, the DO concentration remains same till 66h but after that it started rising again due to depletion of sugar concentration in the medium and approaches around 28% at 72 h. Due to second intermittent dosing of 30% of sucrose solution at 72 h, it has been observed that the DO profile again drops down and reaches less than 5% at 84 h of fermentation cycle. Availability of sufficient sugar concentration in the medium maintains around the same DO concentration till 90 h but after that it started rising again due to depletion of nutrients concentration in the medium and gradually approaches around 20% at the end of the fermentation cycle. During the initial optimization studies using agitation, aeration and temperature with uncontrolled DO and intermittent feeding strategy, it was observed that cells were responding with alteration of parameters which has reflected in cell growth as well as in changes in specific CoQ₁₀ content. There was no adverse effect on growth or metabolite production. The pH was found to incline during initial 24 h and then decline rapidly till 60 h. After 60 h, one more pH drop has been noticed at 84 h due to intermittent dosing of 30% sucrose solution at 72 h. After 84 h, it increased gradually till 108 h and reached around pH 7.15.

Maximum titre (62.7 mg/L) and DCW (35.6 g/L) was obtained at 32°C, 0.3 vvm and 700 rpm using optimized fed batch fermentation strategy. Overall the fermenter optimization studies with respect to temperature, aeration and agitation brought significant (1.67 fold) improvement in CoQ₁₀ titre mostly due to 43 % improvement in DCW, whereas no significant improvement in specific CoQ₁₀ content was observed as compared to shake flask fermentation.

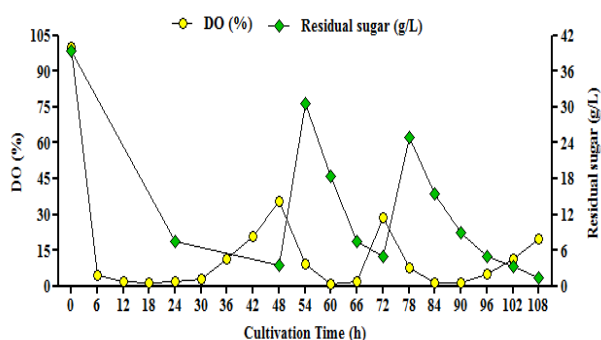


Fig. 6 The generalized DO and residual sugar trend during optimization of temperature, aeration, agitation using fed batch fermentation for PF-P1

F. Optimization of Dissolved Oxygen in Fermenter

DO have a great influence on CoQ₁₀ production using microorganisms. It was reported that cell growth and CoQ₁₀ production were affected by various DO concentrations with *Agrobacterium* sp. and it was also been showed earlier that DO concentration had a great effect on the specific cell growth rate and DCW of *Rhizobium radiobacter* WSH2601 [22]. CoQ₁₀ production was enhanced by limited oxygen supply in *R. radiobacter* suggesting plausible mechanism where the limited electron transfer stimulating the environments of limited oxygen supply and oxidative stress could accumulate the CoQ₁₀ providing the relationship between the CoQ₁₀ physiological functions and its regulation system [23]. Effect of oxygen on CoQ₁₀ production by *Paracoccus denitrificans* was also reported [24]. The pH and dissolved oxygen levels were found to be the key factors affecting CoQ₁₀ productions in *A. tumefaciens* [25]. The optimal oxidation-reduction potential suggested by Sakato et al., (1992) [26] was -150 mV for cell growth and -200 mV for CoQ₁₀ accumulation in cells. In the present study, during optimization of DO condition in fermenter, trials were performed using different DO concentrations like 10%, 20%, 30%, 40% and typical fermenter kinetics at different DO concentrations with respect to CoQ₁₀ titer, DCW and specific content during fermentation cycle. It was observed that 30% DO (of air saturation) showed improvement in biomass build up as well as CoQ₁₀ production than other DO trials. It was observed that DO concentration had a great effect on DCW of strain PF-P1. The optimum DO concentration improved the cell growth and a higher DCW thus obtained. The optimum DO concentration for CoQ₁₀ biosynthesis was 30% of air saturation, and higher or lower DO concentration was less favourable for CoQ₁₀ formation. It has been reported that maximum biosynthesis could be obtained when the oxygen utilization rate was controlled at a suitable level with *Agrobacterium* species [22]. At the condition of 30% DO concentration, the maximal CoQ₁₀ concentration reached 75.49 mg/L, which was much higher than that at other DO concentrations. The maximal specific content also reached the highest value of 1.9892 mg/g of DCW at DO concentration of 30% of air saturation. The comparison in DCW, CoQ₁₀ titre and specific CoQ₁₀ content obtain at different DO concentration control methods by fusant PF-P1 is shown in Table 4.

TABLE 4. Comparison of results at different DO concentration control methods for PF-P1 (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

DO (%) [of air saturation]	Temperature (°C)	Fermentation Cycle (h)	DCW (g/L)	Titer (mg/L)	Specific CoQ ₁₀ Content (mg/g of DCW)
10	32	108	36.2	66.65	1.8440
20	32	108	36.7	70.5	1.9251
30	32	108	38	75.49	1.9892
40	32	108	35.9	64.1	1.7882

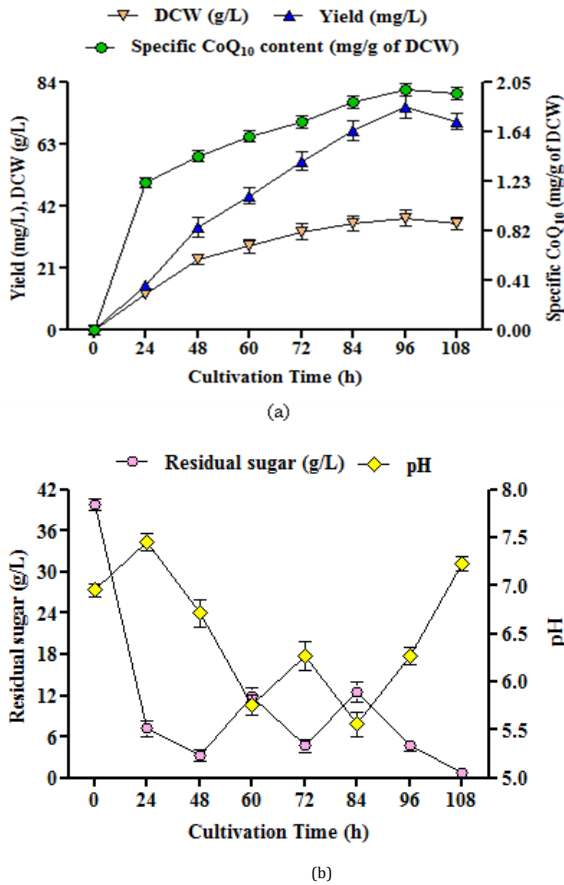


Fig. 7 Time course of fed batch fermentation for PF-P1 at optimized DO condition [parameters like temperature: 32°C, DO 30% (of air saturation)] (a) Kinetics for titre, DCW and specific CoQ₁₀ content during cultivation, (b) kinetics for residual sugar consumption and pH during cultivation (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

The time course of the batch run at 32°C with 30% DO of air saturation is shown in Fig.7. At 30% DO (of air saturation), the highest titre of 75.49 mg/L was observed at 96 h with 38 g/L of DCW and 1.9892 mg/g of DCW keeping temperature 32°C as shown in Fig. 7 (a). A relatively less oxygen availability was needed for cell growth of strain PF-P1. The CoQ₁₀ biosynthesis was improved by optimizing the DO concentration for fusant PF-P1. It has been reported that the DO concentration might have a strong influence on cellular metabolism [27]. High or low DO concentration was not favourable for CoQ₁₀ biosynthesis according to the results obtained. When the DO concentration was controlled at higher level, the intermediates in CoQ₁₀ biosynthesis [28, 29], such as pyruvate, D-glyceraldehyde-3-phosphate, phenylalanine, tryptophan etc. were produced via glycolysis pathway. On the other hand, when DO concentration was at a lower level, saccharide break down was alleviated by TCA cycle [30]. Fig. 7 (b) describes the residual sugar and pH trend obtained during fermenter condition like temperature 32°C along with 30% of DO (of air saturation). The pH was found to incline during initial 24 h and then decline rapidly till 60 h. After 60 h, one more pH drop has been noticed due to dosing of 30% sucrose at 72 h. After 84 h, it increased gradually till 108 h and reached around pH 7.23. This intermittent dosing of 30% sucrose solution at 48 h, 72 h respectively helped in

maintaining the sugar concentration above 7-10 g/L till late of 84 h of fermentation cycle resulted in the biomass build up.

G. Optimization of pH in Fermenter

In the present study, during optimization of pH condition in fermenter, trials have been performed at different pH like 6.0±0.05, 6.5±0.05, 7.0±0.05, 7.5±0.05 respectively, keeping constant parameters like temperature 32°C and DO 30% (of air saturation). The typical fermenter kinetics at different pH conditions with respect to CoQ₁₀ titre, DCW and specific content during fermentation cycle. It was observed that pH had a great effect on CoQ₁₀ production and cell growth of strain PF-P1. The specific CoQ₁₀ content at pH 7.0±0.05 (2.4448 mg/ g of DCW) was much higher than at pH 7.5±0.05 (1.4121 mg/g of DCW), and CoQ₁₀ production was the highest (113.68 mg/L) at pH 7.0±0.05, in spite of the highest DCW (47.7 g/L) occurring at pH 7.5±0.05. At pH 6.0±0.05, DCW and the specific CoQ₁₀ content decreased to 26 g/L and 1.6110 mg/ g of DCW, respectively. At pH 6.5±0.05, DCW and the specific CoQ₁₀ content decreased to 31.7 g/L and 1.6493 mg/ g of DCW, respectively. It was observed that, CoQ₁₀ production was the highest at pH 7.0±0.05, and decreased due to a lower DCW at acidic pH and a lower specific CoQ₁₀ content at alkaline pH. The comparison in DCW, CoQ₁₀ titre and specific content obtain at different pH condition in fermenter using fusant PF-P1 is shown in Table 5.

TABLE 5. Comparison of results at different pH auto-control condition for PF-P1 (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

pH	DO (%) [of air saturation]	Temperature (°C)	Fermentation Cycle (h)	DCW (g/L)	Titer (mg/L)	Specific CoQ ₁₀ Content (mg/g of DCW)
6.0±0.05	30	32	108	26	41.91	1.6110
6.5±0.05	30	32	108	31.7	52.36	1.6493
7.0±0.05	30	32	108	46.4	113.68	2.4448
7.5±0.05	30	32	108	47.7	67.13	1.4121

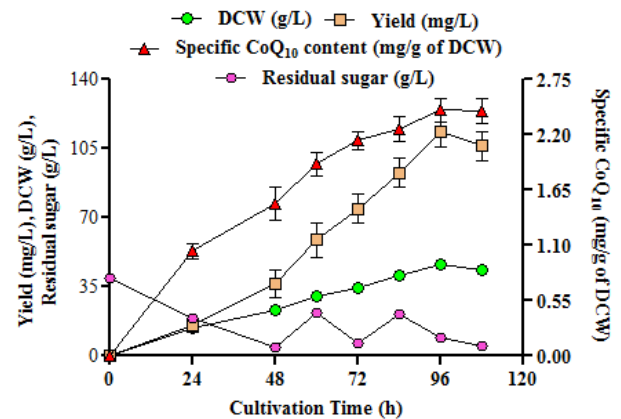


Fig. 8 Time course of fermentation for PF-P1 at optimized pH condition [parameters like temperature: 32°C, DO 30% (of air saturation), pH 7.0±0.05] Kinetics for titre, DCW, specific CoQ₁₀ content and total residual sugar consumption during cultivation (each value represents the mean of triplicate measurements and varied from the mean by not more than 15%)

The time course of the batch run at 32°C, 30% DO of air saturation with auto-control pH 7.0±0.05 is shown in Fig. 8. At pH 7.0±0.05, the highest titre of 113.68 mg/L was observed at 96 h with 46.4 g/L of DCW and 2.4448 mg/g of DCW keeping temperature 32°C and 30% DO (of air saturation).

The CoQ₁₀ biosynthesis was improved by optimizing the pH condition for fusant PF-P1. It has been reported that CoQ₁₀ production was growth associated and pH has a great effect on CoQ₁₀ production using *Rhodobacter sphaeroides*. The CoQ₁₀ production was 1.7 times more than uncontrolled pH condition [31]. This intermittent dosing of 30% sucrose solution at 48 h, 72 h respectively with auto-control pH 7.0±0.05 helped in the biomass build up than the batches run with uncontrolled pH, resulted in better CoQ₁₀ production and accumulation of CoQ₁₀ in the cell. In the present study, maximum titre (113.68 mg/L) and biomass (46.4 g/L) was obtained at 32°C, 30% DO (of air saturation) and pH 7.0±0.05 with intermittent dosing of 25 mg/L of pHBA (final concentration) at 24 h followed by 30% of sucrose solution at 48 h, 72 h respectively.

IV. CONCLUSIONS

A novel fusant strain PF-P1 derived from the mutant strains of wild type *P. denitrificans* ATCC 19367 was utilized for CoQ₁₀ fermentation optimization studies in 2 L laboratory glass fermenter. It was found that a maximum of 46.4 g/L of DCW with 113.68 mg/L of CoQ₁₀ was obtained at 32°C temperature, 30% of DO (air saturation) and pH 7.0±0.05 with specific content of 2.4448 mg/g DCW with intermittent dosing of pHBA at a concentration of 25 mg/L (final concentration) at 24 h followed by 30% of sucrose solution at 48 h and 72 h respectively was useful for biomass build up and subsequent improvement in CoQ₁₀ titre. This optimization showed significant improvement in the production of cell biomass (p-value 0.0003), CoQ₁₀ titre (p-value 0.0007) and specific CoQ₁₀ content (p-value 0.0017).

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