Process and Strain Development for Reduction of Broth Viscosity with Improved Yield in Coenzyme Q$_{10}$ Fermentation by Agrobacterium tumefaciens ATCC 4452

Pradipta Tokdar*, Amit Wani, Pratyush Kumar, Prafull Ranadive and Saji George

Natural Products Department, Fermentation Technology, Piramal Enterprises Ltd, Nirlon Complex, Off Western Express Highway, Goregaon (East), Mumbai-400063, India

Abstract

Viscous nature of the fermentation broth has phenomenal influence on process conditions and parameters in a fermentor. Though broth rheology has attracted significant influence in process research, still there is a challenge to modify fluid dynamics of fermentation broth. During the production of coenzyme Q$_{10}$ (CoQ$_{10}$) by Agrobacterium tumefaciens ATCC 4452, the culture broth becomes highly viscous due to excessive synthesis of exopolysaccharides. This hinders the CoQ$_{10}$ yield and complicates the downstream process. The present study describes how this problem was tackled by media modification and mutation. Induced mutants were generated using UV and EMS as mutagenic agents followed by rational selection based on antibiotic resistance. On screening of these mutants in sucrose based PM-2 medium, UV induced, vancomycin resistant mutant M-6, showed significant reduction (6.29 fold) in viscosity development in the broth. Mutant M-6(S), a natural variant of mutant M-6, resistant to high substrate concentration was further selected for the CoQ$_{10}$ production. Cane molasses as carbon source was found to be best suitable for CoQ$_{10}$ fermentation using mutant M-6(S). Replacing sucrose with cheaper cane molasses significantly reduced the broth viscosity with improved specific CoQ$_{10}$ content, thereby generating cost effective fermentation process. The newly developed mutant strain produced 48.89 mg/L of CoQ$_{10}$ with specific CoQ$_{10}$ content of 1.87 mg/g of DCW at 25°C, 500 rpm agitation and 0.2 vvm aeration using continuous fed batch fermentation and newly formulated cane molasses medium.

Keywords: Agrobacterium tumefaciens; CoQ$_{10}$; Exopolysaccharide; Cane molasses; Viscosity

Introduction

CoQ$_{10}$ is 2,3-dimethoxy-5-methylbenzoquinone with 10 units of isoprenoid chain at the 6-position of the quinone ring. It is distributed widely in the mitochondrial inner membrane, lysosomes, peroxisomes, and microsomes throughout the eukaryotic cells, and is located in the plasma membrane of prokaryotic cells, transferring electrons from complex I/II to the cytochrome bc$_{1}$ complex during the oxidative phosphorylation for ATP generation [1,2]. Apart from playing an important role in electron transport chain for ATP synthesis, it also acts as an antioxidant and prooxidant [3]. It is widely used as neutraceuticals and therapeutical supplements in various clinical conditions like cardiovascular disease, neuro-degenerative diseases, and mitochondrial respiratory-chain diseases etc [4-6]. It is also used as a dietary supplement in energy drinks. Hence, there is burgeoning demand for CoQ$_{10}$ in the global market by neutraceutical companies [7].

CoQ$_{10}$ can be produced by chemical, semi chemical and biological methods [8]. Wild type strains and chemical mutants of various microorganisms, including bacteria (e.g., Agrobacterium, Rhodobacter, Paracoccus) and yeasts (e.g. Candida, Rhodotorula, Saccharomyces) have been reported as CoQ$_{10}$ producers in the patent applications [9]. Among CoQ$_{10}$ producing microorganisms, Agrobacterium tumefaciens has been reported to produce higher CoQ$_{10}$ [10]. But during the biosynthesis, the culture broth becomes highly viscous due to formation of exopolysaccharides when cultivated on sucrose-based medium [11]. This markedly changes the rheological nature of the fermentation fluid, thereby affecting oxygen transfer in fermentor [12]. Also during the downstream processing, the highly viscous broth makes the purification process difficult. Many researchers have addressed the issue at the process level. It was reported that sugar to ammonium salt ratio in the medium as well as temperature affects CoQ$_{10}$ production and viscosity [13]. The effect of sucrose concentration on the production of exopolysaccharide, cell growth and specific CoQ$_{10}$ content has been studied. Using pH stat fed batch culture system with regulation of sucrose concentration; there was a significant reduction in exopolysaccharide with concomitant improvement in CoQ$_{10}$ content [14].

In this paper, we describe a combination of mutations method along with process modification to reduce the broth viscosity for improved CoQ$_{10}$ production by Agrobacterium tumefaciens ATCC 4452.

Materials and Methods

Strains and morphology

The bacterial strain Agrobacterium tumefaciens ATCC 4452 and its induced mutants were maintained on Nutrient Agar (NA) slants. The mutants were streaked on NA plates and incubated at 30°C for 2-3 days in order to get the pure clone.

*Corresponding author: Pradipta Tokdar, Natural Products Department, Fermentation Technology, Piramal Enterprises Ltd, Nirlon Complex, Off Western Express Highway, Goregaon (East), Mumbai-400063, India, Tel: (+91) 22 30818840; Fax: (+91) 2230818036; E-mail: pradipta.tokdar@piramal.com

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UV mutagenesis

The culture was grown overnight in 50 ml of nutrient broth (NB) medium in 500 ml flask to get the exponential phase growth culture having viable count of around 10^8-10^11 cfu/ml. Five ml of the suspension was placed in sterile petri dish and exposed to UV rays (235 nm) at a distance of 10 cm. At regular intervals, the samples were taken out and different dilutions were plated on NA plate to determine viable count. The percent reduction of viability was calculated by comparing viable count with that of unexposed suspension.

EMS mutagenesis

10 ml suspension of exponential phase growth culture having viable count of around 10^8-10^11 cfu/ml was centrifuged to get a pellet. It was then washed with saline and resuspended in 10 ml of phosphate buffer (pH 7.0). The suspension was treated with 80 μM of EMS with constant shaking. At different time intervals samples were withdrawn and to it 5% sodium thiosulphate was added to stop the mutagenesis. Next the cells were washed and plated on NA plate to determine the viable count. The reduction in viable count was determined as compared to untreated suspension.

Selection of mutants

The mutant showing resistance to antibiotic was selected. Initially antibiotic sensitivity test was performed using different antibiotic discs (Octadisc, Hi-media) by agar diffusion method.

Vancomycin to which the strain was most sensitive was used for selection of resistant mutants by gradient plate technique using vancomycin from 0-60 μg/ml. The plates were incubated at 30°C for 96 h. The mutant colonies appearing towards vancomycin concentration above MIC (30 μg/ml) were picked up and transferred onto NA slants.

Screening of mutants and media modification in shake flask

The wild type strain and mutants were inoculated in 50 ml seed medium containing (per L) 20 g sucrose, 10 g yeast extract, 10 g peptone, 5 g NaCl, pH 7.2 in a 500 ml Erlenmeyer flask and incubated at 30°C with shaking at 220 rpm. After 48 h, 10% of the seed was transferred to 50 ml of different production media in 500 ml Erlenmeyer flask [15]. The production media used [15-18] were PM-2 (consists of (per L) 25 g sucrose, 10 g (NH₄)₂ SO₄, 2.5 g K₂HPO₄, 2 g KH₂PO₄, 0.5 g MgSO₄.7H₂O, 20 g corn steep liquor (CSL), 20 g CaCO₃, 1 ml/L trace element solution, pH 7.0), MPM-1 (consists of (per L) 50 g sucrose, 10 g (NH₄)₂ SO₄, 2.5 g K₂HPO₄, 2 g KH₂PO₄, 0.5 g MgSO₄.7H₂O, 20 g CSL, 20 g CaCO₃, pH 7.0), MPM-2 (consists of (per L) 30 g sucrose, 1 g (NH₄)₂ SO₄, 2.5 g K₂HPO₄, 2.5 g KH₂PO₄, 0.5 g MgSO₄.7H₂O, pH 7.0), MPM-3 (consists of (per L) 80 g sucrose, 100 g cane molasses, 13 g (NH₄)₂ SO₄, 2.5 g K₂HPO₄, 2 g KH₂PO₄, 0.5 g MgSO₄.7H₂O, 40 g CSL, 20 g CaCO₃, pH 7.2), MPM-4 (consists of (per L) 50 g dextrose, 10 g soyabean meal, 5 g yeast extract, 4 g soya peptone, 2.5 g NaCl, 50 g CaCO₃, pH 7.2), MPM-5 (consists of (per L) 80 g cane molasses, 13 g (NH₄)₂ SO₄, 2.5 g K₂HPO₄, 2.5 g KH₂PO₄, 0.5 g MgSO₄.7H₂O, 40 g CSL, 20 g CaCO₃, pH 7.2), MPM-6 (consists of (per L) 50 g cane molasses, 10 g (NH₄)₂ SO₄, 2.5 g K₂HPO₄, 2.5 g KH₂PO₄, 0.5 g MgSO₄.7H₂O, 20 g CSL, 20 g CaCO₃, pH 7.0) and MPM-7 (consists of (per L) 80 g sucrose, 13 g (NH₄)₂ SO₄, 2.5 g K₂HPO₄, 2 g KH₂PO₄, 0.5 g MgSO₄.7H₂O, 40 g CSL, 20 g CaCO₃, 1 ml/L trace element solution, pH 7.0). The production flasks were incubated at 30°C with shaking at 220 rpm for 90-96 h.

The sucrose based media namely PM-2, MPM-1, MPM-3 and MPM-7 were dosed intermittently at 48 h and 72 h with 5 ml of 60% cane molasses solution. The glucose based media namely MPM-2 and MPM-4 were dosed intermittently at 48 h and 72 h with 5 ml of 60% glucose solution.

Lab scale fermentation

The performance of mutant M-6(S) in four different media namely PM-2, MPM-3, MPM-5 and MPM-7 was evaluated in 10 L NBS fermentor. The fermentation was carried out at 30°C with 300 rpm and 0.3vvm aeration. The sucrose based media namely PM-2, MPM-3 and MPM-7 were dosed intermittently at 48 h and 72 h with 500 ml of 30% sucrose solution. Dosing was carried out as per the published protocol [12,15]. The cane molasses based media namely MPM-5 was dosed intermittently at 48 h and 72 h with 500 ml of 60% cane molasses solution. The fermentor was harvested at 96 h and broth viscosity, specific CoQ₁₀ content and DCW was estimated.

Viscosity measurement

The viscosity of 35 ml of each of the broth samples was estimated using Vibro Viscometer SV-10 and expressed as cps unit.

Dry cell weight (DCW) measurement

10 ml of broth was centrifuged at 12000 rpm for 20 min in a pre-weighed centrifuge tube. The cell mass was quantified by drying at 60°C until a constant mass.

CoQ₁₀ extraction method

The CoQ₁₀ extracted from cell biomass was quantified on High Performance Liquid Chromatography (Agilent 1100) using normal phase Kromasil silica column (250 mm×4.6 mm, 5 μ particle size) and hexane: isopropyl alcohol (95:5) as mobile phase with a flow rate of 1ml/min. Detection was carried out at 273 nm.

Estimation of total sugar

Total sugar of the fermentor broth has been estimated by Anthrone method [19].

Feasibility optimization

The fermentation process was optimized for mutant M-6(S) using MPM-5 medium in 10 L NBS fermentor. A 10% (v/v) seed culture was inoculated into a 10 L fermentor with a working volume of 7 L. The fermentation was done by altering the parameters like temperature (25°C, 30°C and 30°C), agitation (300, 500 and 600 rpm) and aeration (0.2 and 0.6 vvm) [20-22]. The continuous feeding was started after 24 h of growth with 20.5% cane molasses solution at the flow rate of 10 ml/h. The batch was harvested at 96 h. The broth samples were analyzed for DCW and specific CoQ₁₀ content.

To study the effect of sucrose on viscosity development in the broth, a fermentor batch was carried out using the optimized parameters and replacing the cane molasses in the medium (MPM-5) by equivalent amount of sucrose (4%). The dosing was started after 24 h of growth with 10.25% sucrose solution at the flow rate of 10 ml/h. The batch...
was harvested at 96 h. The broth samples were analyzed for DCW and specific CoQ<sub>10</sub> content.

**Statistical analysis**

For analyzing 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 3 trials. Unless stated otherwise, all error bars represent standard error of mean.

**Results and Discussion**

The wild type strain of *Agrobacterium tumefaciens* ATCC 4452 exploited for production of CoQ<sub>10</sub> produced excessive amounts of viscous exopolysaccharide on sucrose based medium, which complicated its downstream processing an affected CoQ<sub>10</sub> yield. To tackle the issue of exopolysaccharide production, type strain was subjected to genetic manipulation by physical and chemical mutagenesis followed by rational selection of mutants, probably lacking polysaccharide biosynthetic pathway resulting in reduced broth viscosity.

The wild type strain showed more than 95% reduction in viability when exposed to germicidal UV rays for 7 min at a distance of 10 cm. In case of EMS mutagenesis, about 94% reduction in viability was observed when the cells were treated with 80 μM EMS for 30 min. The selections of mutants were carried out based on the resistance to the different antibiotic and finally vancomycin was selected to which strain was found to be most sensitive among all tested antibiotics using octadisc agar diffusion method. The MIC of vancomycin was found to be around 30 μg/ml using gradient plate technique.

Screening of selected mutants from gradient plate which were growing beyond the MIC (30 μg/ml) of vancomycin has been performed in shake flask using sucrose based PM-2 medium. Among the different mutants selected and tested, mutant M-6 showed 6.29 fold decreases in viscosity and 1.27 fold increases in specific CoQ<sub>10</sub> content as compared to wild type strain, as shown in figure 1. Drop in broth viscosity was exploited for production of CoQ<sub>10</sub>, produced excessive amounts of mean.

Attempts were made to formulate new media for CoQ<sub>10</sub> production using mutant M-6(S). We formulated completely a new media MPM-5 containing combination of sucrose and cane molasses. In addition to this, MPM-3 containing combination of sucrose and cane molasses was formulated. During shake flask screening, intermittent dosing with the respective carbon source was carried out for each of the medium flasks at log 48 and 72 h to maintain the total sugar concentration above 6%. This feeding helped to achieve extended growth phase and improved biomass yield.

Out of seven media tested in flask, medium MPM-5 containing 8% cane molasses showed maximum specific CoQ<sub>10</sub> content (1.398 mg/g of DCW) and least viscosity of broth (3.766 cps) as shown in figure 2. This reduction of broth viscosity and improvement of specific CoQ<sub>10</sub> content was significant with medium MPM-5 as compared to PM-2 medium. The medium MPM-3 and MPM-6 also showed significant reduction in broth viscosity without any significant change in specific CoQ<sub>10</sub> content. Since the profile of these two media was identical, only MPM-3 was selected for further studies. In addition to these, medium MPM-7 containing higher sucrose was also selected for further studies in order to compare the performance between two carbon sources i.e., molasses and sucrose.

From the shake flask experiments, we finally selected four media namely initial medium PM-2 (25 g sucrose per L), MPM-3 (combination of 80 g sucrose and 100 g cane molasses per L), MPM-5 (80 g cane molasses per L), and MPM-7 (80 g sucrose per L) to study the

![Figure 1: Comparison of viscosity and specific CoQ<sub>10</sub> content between wild type and mutant M-6 on PM-2 medium at shake flask level.](image1.png)

![Figure 2: Comparison of viscosity and specific CoQ<sub>10</sub> content of mutant M-6(S) with different production media at shake flask level.](image2.png)
performance of mutant M-6(S) in these media at lab scale fermentor level under identical fermentation conditions e.g., temperature 30°C, agitation 300 rpm and aeration 0.3 vvm. The specific CoQ<sub>10</sub> content and the viscosity obtained with four media in fermentor are shown in figure 5.

Although mutant M-6(S) did not show much viscosity development in the shake flask with any of the tested media, the significant development of broth viscosity was observed in the medium containing sucrose as sole carbon source (PM-2 and MPM-7) at fermentor level. The combination of sucrose and cane molasses did not induce viscosity development in fermentor as seen from medium MPM-3. The significant rise in specific CoQ<sub>10</sub> content 1.37 mg/g of DCW was achieved by the medium MPM-5 containing cane molasses as sole carbon source in fermentor; however there was no viscosity development. The results indicate that sucrose in the media is a major contributing factor for viscosity development in mutant M-6(S) under accelerated fermentation condition. In addition to this, the cane molasses, a by-product of sugar processing industries, is much cheaper source than sucrose, thus contributing towards cost effective fermentation process.

Hence, it can be concluded that mutant M-6(S) is a promising strain for efficient and cost effective production of CoQ<sub>10</sub> using MPM-5 medium. Further the optimization of fermentation conditions for mutant M-6(S) were carried out in 10 L laboratory scale fermentor using cane molasses based newly formulated MPM-5 medium.

The optimization was done with respective aeration, agitation and temperature. During fermentation of mutant M-6(S) on cane molasses medium, feeding was carried out using 20.5% of cane molasses solution from 24 h at the flow rate of 10 ml/h. The total sugar concentration dropped from 12% to 8% and then it was maintained at around 8% till end with the help of continuous dosing. The same fed batch strategy was adopted for further optimization in fermentor by altering the parameters. The specific CoQ<sub>10</sub> content, DCW, and CoQ<sub>10</sub> titer obtained with different fermentation conditions are expressed in table 1. The maximum specific CoQ<sub>10</sub> content (1.87 mg/g of DCW) was achieved with 500 rpm agitation, 0.2 vvm aeration and temperature of 25°C. It was observed that lowering the aeration from 0.6 vvm to 0.2 vvm helped in almost 1.36 fold improvement in specific CoQ<sub>10</sub> content. The temperature of 25°C was found to be optimum for the CoQ<sub>10</sub> process that showed maximum CoQ<sub>10</sub> content.

Figure 4 shows the kinetics of the fermentor batch carried out with optimized process parameters using cane molasses medium. The figure 5 shows the HPLC chromatogram of standard CoQ<sub>10</sub>. The HPLC chromatogram of the crude extract from mutant M-6(S) indicating the presence of CoQ<sub>10</sub> peak is shown in figure 6.

There was not much fluctuation in pH throughout the cycle and it was automatically maintained in the range of 7.2 to 7.7 due to continuous dosing. During the initial 24 h of growth phase, there was rapid built up of biomass and sudden drop in DO. At 24 h, the continuous feeding was started to maintain the total sugar concentration at 8% throughout the cycle. Due to initiation of continuous dosing, DO have started rising and maintained in the range of 50-60% till the end of the cycle. There was a linear rise in CoQ<sub>10</sub> titer with biomass and reached maximum of 48.89 mg/L at the end of the process. From the process, it is observed that the exponential phase of the culture is maintained till the end with the help of continuous dosing. The optimized process parameters, fermentation medium and continuous dosing has helped to maintain and prolong the exponential growth phase, resulting in higher CoQ<sub>10</sub> titer and biomass.

It was thought to check the performance of sucrose-based media under optimized fermentation condition with mutant M-6(S). In the fermentor, cane molasses was replaced with equivalent quantity of pure sucrose and sucrose was used as a dosing solution. The comparison of these two carbon source are shown in figure 7, where we found a linear rise in viscosity of the broth reaching up to 128 cps at the end of fermentation for sucrose containing medium. The viscosity was significantly higher, than observed in cane molasses media. It was significantly drop in specific CoQ<sub>10</sub> content 1.37 mg/g of DCW was achieved by the medium MPM-5 containing cane molasses as sole carbon source (PM-2 and MPM-7) at fermentor level under identical fermentation conditions e.g., temperature 30°C, agitation 300 rpm and aeration 0.3 vvm. High sucrose containing MPM-7 medium showed significant rise in viscosity (p-value 0.0018) as well as significant drop in specific CoQ<sub>10</sub> content (p-value 0.0040). For statistical analysis, student’s t-test was used using PRISM-5 software, p-value below 0.05 was considered statistically significant. The values in the graph are an average of 3 trials. All error bars represent standard error of mean.
Figure 5: HPLC chromatogram of standard CoQ₁₀.

Figure 6: HPLC chromatogram of crude extract.
also observed that on sucrose medium after 72 h of cycle, CoQ_{10} titer decreased to 1.9 fold with increasing viscosity, which might be due to poor mass transfer in fermentor. The wild type strain showed more than 400cps viscosity when grown on this medium in the fermentor. In case of the mutant strain M-6(S), although the viscosity development on sucrose medium was less than that obtained with wild type strain, it was still higher than viscosity observed on the sole molasses medium. Thus it can be concluded that sucrose is a major contributing factor for viscosity development in broth, due to induction of polysaccharide biosynthesis and hence not a suitable carbon source for CoQ_{10} process using Agrobacterium tumefaciens. Mutant strain M-6(S) showed substantial improvement over wild type strain in terms of maintaining broth rheology under accelerated fermentation condition, hence it could be exploited further for CoQ_{10} bioprocess. It would be worth investigating the changes occurred at molecular level in mutant M-6(S) especially on exopolysaccharide biosynthesis pathway.

Conclusion

Development of enormous viscosity of the broth, due to exopolysaccharide production, was a major hurdle faced during CoQ10 fermentation process using Agrobacterium tumefaciens ATCC 4452 wild type strain, which not only affected the yield, but also made the bio-separation process difficult. Attempts were made to overcome this problem by developing a suitable mutant strain and production medium for fermentation process, which resulted in reduction of broth viscosity and higher content of CoQ10. The newly developed mutant strain mutant-6(S) produced 48.89 mg/L of CoQ10 with specific CoQ_{10} content 1.87 mg/g of DCW at 25C, 500 rpm and 0.2vvm in the continuous fed batch fermentation using newly formulated cane molasses based medium MPM-5. Replacing sucrose in the fermentation medium with cheaper cane molasses helped in improving CoQ10 content without producing viscous exopolysaccharide and thus made the process simpler and cost effective. The sucrose was found to be the contributing factor in development of viscosity in Agrobacterium tumefaciens ATCC 4452. Re-mutation of existing mutant and evaluation of new mutant strains at fermentor level with additional nutrient supplementation may lead to economic CoQ_{10} bioprocess.

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