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# Mutagenesis Induced Improvement of Coenzyme Q10 Production by *Agrobacterium tumefaciens*

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**ABSTRACT**

**Mutagenesis induced improvement of coenzyme Q10 production by *Agrobacterium tumefaciens* with the view of increasing CoQ10 production via strain development by UV and EMS mutagenesis is of great importance. Further optimisation of fermentation parameters such as pH, temperature, and inoculum load was done to maximise CoQ10 yield. Soil and gall samples were collected from vegetable field of Research farm located at Chatha, Jammu. Repeated isolations and purification resulted in 05 *Agrobacterium tumefaciens* cultures from soil samples and 07 from that of gall samples. Identification of cultures was confirmed by morphological studies. *Agrobacterium tumefaciens* cultures were grown on selective media to screen CoQ10 producing strains. G12 was best amongst 12 isolates; it produced 2.36 mg/g DCW. Mutagenesis using UV and EMS treatment was done to obtain a high-CoQ10-producing strain from the native isolate (G12). When exposed for 7 minutes to mutagenic UV radiation from a distance of 10 cm, the native strain (G12) showed reduction in number of colonies. Also the viability of cells was reduced when they were treated with 80 µM EMS for 30 min but it was less effective than UV mutagenesis. G12 UV mutant strain was selected after screening and tested for CoQ10 production potential by flask culturing. Our results indicate that CoQ10 content increased from 2.36 mg/g DCW to 4.34 mg/ g DCW after mutagenesis, indicating positive mutations. G12 UV mutant was further studied in batch cultures with different inoculum loads at various temperatures and range of pH, to maximize CoQ10 production along with constant fermentation parameters like agitation (180 rpm) and incubation time (96 hours). Therefore, this study suggests that amongst the culture conditions tested so far for CoQ10 production, G12 mutant strain showed maximum CoQ10 content when $10^8$ CFU/ml of inoculum load is used at 32°C and pH 7.0.**

***Keywords-*** *Agrobacterium tumefaciens*, CoQ10, mutagenesis, fermentation parameters.

## I. INTRODUCTION

The substance that improves the action of an enzyme is known as coenzyme. Coenzymes are tiny molecules that play an important role in the human body. They cannot catalyse reactions on their own, but they can assist enzymes in doing so. Coenzymes are organic non protein molecules that bind to the protein molecule (apoenzyme) to create the active enzyme (holoenzyme). Ubiquinone is another name for Coenzyme Q10 (CoQ10). It is typically found in plants, animals, and cells of microorganisms. CoQ10 was first discovered in 1957 by Professor Fredrick L. Crane and his co-workers at the University of Wisconsin–Madison Enzyme Institute (Turunen *et al.*, 2004). In 1958, Dr. Karl Folkers and Co-workers at Merck reported its chemical structure.

In 1961 Peter Mitchell put forward the concept of electron transport chain which includes the vital proton motive role of CoQ10. He also received a Nobel Prize for the same in 1978. The redox functions of CoQ10 in cellular energy production and antioxidant protection are based on the capability to exchange two electrons in a redox cycle between ubiquinol (reduced CoQ10) and ubiquinone (oxidized CoQ10). Lars Ernster investigated the molecule's antioxidant action as a free radical scavenger in depth. Following that, a number of experts from around the world began studying this

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chemical in relation to a variety of ailments, including cardiovascular disease and cancer.

The primary building block of CoQ10 is tyrosine, which is synthesized in the intracellular region in the human body. Because pyridoxal 5'-phosphate (vitamin B6) is required as a cofactor in the first step, adequate vitamin B6 nutrition is critical for CoQ10 production. Certain circumstances can interfere with the body's ability to produce enough CoQ10 to meet its requirements (Lee *et al.*, 2017). Cells and tissues that are metabolically active have the highest CoQ10 requirements such as the heart, immune system, and gingiva and are thus the most sensitive to CoQ10 shortage.

CoQ10 is a component of the electron transport chain that participates in aerobic cellular respiration and produces energy in the form of ATP. This is how the human body generates 95% of its energy (Pahari *et al.*, 2016). Thus, CoQ10 concentrations are highest in organs that demand more energy, such as the heart, liver, and kidney. It plays an important function in the generation of cellular energy and the scavenging of free radicals in the human body (Ernster and Dallner, 1995). CoQ10's ability to exist in both fully oxidised (ubiquinone) and fully reduced (ubiquinol) forms makes it easier for it to accomplish its functions in the electron transport chain (Pahari *et al.*, 2016). CoQ10 works as an antioxidant and has therapeutic applications in several diseases such as breast cancer (Portakal *et al.*, 2000), heart diseases (Singh *et al.*, 1999), Alzheimer's and Parkinson's diseases (Beal, 2004). Because of its qualities that aid in the prevention of ageing and cardiovascular diseases, CoQ10 has received a lot of attention as a nutraceutical dietary supplement in recent years (Tian *et al.*, 2010).

CoQ10 has many uses in pharmaceutical, confectionary and cosmetics industries. All these applications have led to interest in increasing CoQ10 production to full fill ever-increasing demands. CoQ10 is produced biologically by a variety of microorganisms, including photosynthetic bacteria and yeasts. *Agrobacterium tumefaciens*, *Paracoccus denitrificans* and *Rhodobacter sphaeroides* are also known for synthesizing great amount of CoQ10 (Yoshida *et al.*, 1998). Various attempts have been made to meet the increasing demands of CoQ10 production. CoQ10 is produced from one of the three routes: chemical synthesis, biological tissues and microbial fermentation (Tian *et al.*, 2010; Kawamukai, 2002).

Solanesol is substrate and source for isoprenoid tail during the chemical synthesis of CoQ10 (Mu *et al.*, 2011). There are several other expensive inputs for the production of CoQ10 in addition chemical waste is also generated (Sheldon, 2014; Murphy, 2011; Wenda *et al.*, 2011; Lee *et al.*, 2017). Chemicals and solvents used for its manufacture are detrimental for our environment; therefore first two methods are not preferred. Microbial fermentation is an environmental friendly method involving enzymatic reactions at cellular level for the production of CoQ10. (Murphy, 2011; Du *et al.*, 2011). Cell has its own mechanisms to avoid complexities of synthesis of CoQ10 (Nielsen *et al.*, 2014; Pscheidt and Glieder, 2008). Many researchers have also made an effort to optimize the fermentation parameters such as temperature, carbon/nitrogen ratio, pH, inoculum load etc. with the possibility of increasing CoQ10 productivity. Microbial processes, metabolic engineering strategies are employed to increase the titer of CoQ10 and in order to decrease steps involved during its biosynthesis. Chemical mutagenesis-based selection and chemical engineering processes that focused on modifying substrate flux were initially utilised in metabolic engineering approaches; however, the discipline has since evolved to encompass other genetics-based strategies (Murphy, 2011; Lee *et al.*, 2012). Flux improving is the best and simplest method for increasing CoQ10 yield (Murphy, 2011; Jullesson *et al.*, 2015). Chemical obstructions responsible for restricting product development can be overcome by using genes that reconstitute cofactors, such as NADPH and S-adenosyl methionine (SAM) (Lee *et al.*, 2012; Brown *et al.*, 2015) Overall, it is obvious that comprehensive examination and adjustment of biosynthetic pathways can direct and optimise metabolic flow.

CoQ10 is only indigenous to a few organisms (Kawamukai, 2009; Wang and Hekimi, 2013) and it is not known whether human metabolic reactions can cope with a less CoQ10 concentrations (Okada *et al.*, 1998; Hihi *et al.*, 2003). Traditionally, the majority of efforts have been focused on native CoQ10 producers and mutant strains with increased CoQ10 production. However, converting heterologous hosts with a large toolbox, such *as Escherichia coli* and *Saccharomyces cerevisiae*, into platforms for CoQ10 synthesis has a lot of potential.

Several workers have reported CoQ10 production by local isolates such as *Schizosaccharomyces pombe*, *Rhodobacter sphaeroides* and *Agrobacterium tumefaciens* (Dixson *et al.*, 2011; Lu *et al.*, 2015; Tokdar *et al.*, 2013). Microorganims like *Paracoccus* bacteria, *Pseudomonas*, *Saitoella* yeasts and *Candida* manufacture CoQ10 indigenously. *Agrobacterium tumefaciens* has best potential to produce CoQ10, as per reports till now (Ha *et al.*, 2007). The output of CoQ10 in liquid cultivation using the wild-type strain of *Agrobacterium tumefaciens* is limited, due to its low specific CoQ10 concentration. Strain development improvement is necessary step for increasing yields of CoQ10 content of *Agrobacterium tumefaciens* (*Yuan et al., 2012*). Strain improvement, followed by optimization of fermentation parameters like C/N ratio, temperature, pH, viscosity and oxygen supply, which ultimately are responsible for improving CoQ10 production.

In present study high CoQ10 producing strain of *Agrobacterium tumefaciens* was generated via strain

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development by UV and EMS mutagenesis. Further optimisation of fermentation parameters such as pH, temperature, and inoculum load was done to maximise CoQ10 yield.

## II. MATERIALS AND METHODS

### *2.1 Collection of samples*

Gall sample was collected from the vegetable field of Research farm located at Chatha, Jammu. 100g of soil from the surrounding area was also collected for isolation of *Agrobacterium tumefaciens*. The samples were brought to the laboratory in sterile polythene bags with great care to avoid contamination and stored in refrigerator at 4°C for further use.

Standard culture of *Agrobacterium tumefaciens* A(609) to be used as control was procured from IMTECH-Chandigarh. This culture was revived on Mannitol agar slants at 27 ± 1° C, and fully grown slants were stored in refrigerator at 4°C. This culture was used for further studies.

### *2.2 Isolation and Characterization*

#### 2.2.1 Isolation of *Agrobacterium tumefaciens* from soil sample

1g of soil sample and 9ml of sterile distilled water were taken as stock. Then serial dilution was performed ranging from $10^{-1}$ to $10^{-9}$. Yeast Extract Mannitol agar is widely used for the cultivation of *Agrobacterium* species. A loopful of sample was collected from the serially diluted culture tubes and inoculated into the prepared YEM plates and kept for incubation at 27 ± 1°C for 2 days. Then, the isolated single colonies were maintained at Mannitol agar slants and were stored in refrigerator at 4°C for further use (Mary *et al.*, 2017).

#### 2.2.2 Isolation of *Agrobacterium tumefaciens* from gall sample

For surface sterilization, firstly the crown gall sample was rinsed with tap water. A solution of 10% commercially available bleach was prepared. For 3-5 minutes, the gall sample was submerged in the solution. To remove any remnants of the bleach solution, the gall sample was rinsed with sterilized distilled water. The gall sample was then cut into little pieces and dried for two to three days (Ali *et al.*, 2016). Then powder was made and stored in sterilized bottle for further use. Stock solution was made by adding 1g of powdered gall sample and 9ml of sterile distilled water in a culture tube. Then serial dilution was performed ranging from $10^{-1}$ to $10^{-9}$ and culture tubes were labelled properly. Loopful of sample was taken from the serially diluted culture tubes and inoculated into the prepared YEM/MacConkey plates and kept for incubation at 27 ± 1° C for 2 days. Then, the isolated single colonies were maintained at Mannitol agar slants and were stored in refrigerator at 4°C for further use (Islam *et al.*, 2010).

#### 2.2.3 Purification

To obtain a pure culture of *Agrobacterium tumefaciens*, the initial bacterial culture was sub cultured on YEM/ MacConkey media. During sub culturing, a single colony was picked from each plate with the help of loop and inoculated into YEM/ MacConkey media using the Streak plate method. The technique was repeated continuously, yielding single colonies on each plate. The petri plates were kept in the incubator for two to three days at 27 ± 1°C after sub culturing. The single colonies of cultures were then transferred to new Mannitol agar slants and stored in refrigerator at 4°C for further studies.

#### 2.2.4 Characterization

##### 2.2.4.1 Gram's Staining

The slide was washed with 95% ethanol. A drop of distilled water was placed on a slide. Then bacterial colony was picked from the bacterial culture, placed on the slide and properly mixed. The smear was heat fixed. Crystal violet dye was applied to the slide with the help of dropper for 30 sec. The slides were rinsed with sterile water to remove the excess dye. Gram Iodine was applied to the slide for 1 min and washed. Then 95% ethanol was applied and rinsed the slide again with the distilled water. The slides were further treated with Safranin called counter stain for 1 min and rinsed (Ali *et al.*, 2016). After drying it was observed under 100X magnification by using a light microscope with the aid of immersion oil.

##### 2.2.4.2 Colony Characterization

Bacterial samples were picked by a loop and inoculated into the YEM agar media by Streak plate method. After streaking, the petri plates were kept in the incubator for two to three days at 27 ± 1°C. Colony characteristics such as texture colour and margins of bacterial cultures were observed and noted down.

### *2.3 Screening and Quantification of Isolates for the ability to produce Coenzyme Q10*

#### 2.3.1 Screening of Isolates for the ability to produce Coenzyme Q10

To achieve the exponential growth phase, the bacterial cultures were grown overnight in 50 mL of nutrient broth (NB) media in a 250 mL flask. The cell suspensions were inoculated on selective medium plate containing 3g/L beef extract, 3g/L yeast extract, 10 g/L peptone, 5g/L glucose, 20 mg/L sodium azide and 0.2 g/L $MgSO_4.7H_2O$ . Later it was incubated for 2 days at 27 ± 1°C (Yuan *et al.*, 2012). The fastest growing colony indicating maximum production of CoQ10 was chosen for further studies.

#### 2.3.2 Quantification of Coenzyme Q10 production

##### 2.3.2.1 Preparation of seed culture

Seed medium required for initiating fermentation was made up of 10 g/L glucose, 5 g/L yeast extract, 5 g/L peptone and 5 g/L NaCl. One loop of bacterial cells grown on a slant overnight was inoculated into seed media and incubated at 32°C for 24 hours on a rotary shaker at 180 rpm (Yuan *et al.*, 2012).

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**2.3.2.2 Fermentation**

Basal fermentation medium was made up of 20 g/L glucose, 10 g/L peptone, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 10 g/L yeast extract, 0.5 g/L $K_2HPO_4$, 0.5 g/L $KH_2PO_4$ 0.5 g/L. $10^8$ CFU/ml of seed culture was transferred into a 250 ml flask with 100ml fermentation medium. The cell count of $10^8$ CFU/ml is determined by using McFarland's standards by adjusting the turbidity of the microbial suspension. For $10^8$ CFU/ml the suspension was compared to the 0.5 McFarland standards and the OD was recorded at 600nm. Temperature, agitation speed and time for fermentation were 32°C, 180 rpm and 96 hours, respectively. With the addition of 3 M NaOH or 2 M HCl, the pH was kept at 7.0 ± 0.1.

**2.3.2.3 Dry cell weight (DCW) measurement**

In a pre-weighed centrifuge tube, 10 ml of broth was centrifuged at 12000 rpm for 20 minutes. The cell mass was determined by drying at 60°C until it reached a constant mass.

**2.3.2.4 CoQ10 extraction**

CoQ10 was extracted from the cell pellet obtained after centrifuging 10 ml of broth at 50°C for 3 hours using an ethanol hexane solvent system (1:1). Following the extraction step, 5 mL of water was used for separation. The hexane layer containing CoQ10 was concentrated and analysed further (Tokdar *et al.*, 2013).

**2.3.2.5 Spectrophotometer analysis**

CoQ10 was identified and quantified by known concentrations of authentic CoQ10 standard (Sigma-Aldrich). Cell mass was estimated using a calibration curve made from a relationship between optical density (OD) at 620 nm and dry cell weight. The CoQ10 measurement was carried out in triplicate.

***2.4 Strain improvement by random mutagenesis***

**2.4.1 UV mutagenesis**

To achieve the exponential growth phase, the culture was grown overnight in 50 mL of nutrient broth (NB) media in a 250 mL flask. 5 ml of suspension was taken in a sterile petri dish and was exposed to UV rays (235 nm) from 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 reduction of viability of cells was observed by comparing viable count with that of unexposed suspension (Tokdar *et al.*, 2013).

**2.4.2 EMS mutagenesis**

To obtain a pellet, a 10 ml suspension of exponential phase growth culture was centrifuged. It was then re-suspended in 10 mL of phosphate buffer after being rinsed with saline (pH 7.0). With continual shaking, the suspension was treated with 80 µM of EMS. To stop the mutagenesis, samples were taken at different intervals and 5% sodium thiosulphate was added. The cells were washed and plated on a NA plate to determine the viable count. The drop in viable count was observed when compared to an untreated suspension (Ranadive *et al.*, 2011).

***2.5 Screening for high CoQ10 producing mutant***

The cell suspensions were inoculated on selective medium plate and incubated for 2 days at 27 ± 1°C. Fastest growing colony which indicates maximum CoQ10 production was chosen and fermented to check the variance in CoQ10 content after mutation. Extraction and quantification of CoQ10 by spectrophotometer as mentioned above.

***2.6 Optimization of CoQ10 production in shake flasks***

Fermentation was carried out using shake flasks. Classical One factor at one time method was used for studying fermentation parameters, where only one factor was kept varied while others were kept constant. For fermentation $10^8$ CFU/ml of inoculum was transferred to a 250ml flask containing 100 ml of fermentation medium and incubated at 32°C and 180 rpm for 96 hours (Ha *et al.*, 2007). With the addition of 3 M NaOH or 2 M HCl, the pH was kept at 7.0 ± 0.1. Three fermentation parameters were determined during the study i.e. pH, temperature and inoculum load. Three levels of the factors were evaluated i.e., low, middle and high for each parameter. All the experiments were performed in triplets. Extraction and quantification of each sample was done to check the CoQ10 content.

## III. RESULTS

***3.1 Isolation and identification of Agrobacterium tumefaciens***

**3.1.1 Isolation of *Agrobacterium tumefaciens***

Isolation of *Agrobacterium tumefaciens* was done from soil/ gall sample collected from the vegetable field at Chatha, Research farm SKUAST- Jammu. Total twelve bacterial cultures (five from soil and seven from gall sample) were isolated from the samples (Table 1).

On YEM agar media, isolated single colonies were white to cream colour, smooth, mucoid, glistering circular with entire edges and on MacConkey media, the shape of the bacterium was convex, colour of the bacteria was pink to brick red and the texture showed that colonies were smooth, circular, mucoid, translucent and had glossy appearance. All the observed characteristics were similar to that of *Agrobacterium tumefaciens* culture.

**Table 1. *Agrobacterium tumefaciens* isolated from soil/ gall sample**

| S. No | Soil | S. No | Gall |
|---|---|---|---|
| 1 | S2 | 1 | G3 |
| 2 | S3 | 2 | G5 |
| 3 | S6 | 3 | G6 |
| 4 | S7 | 4 | G7 |
| 5 | S8 | 5 | G9 |
| | | 6 | G12 |
| | | 7 | G13 |

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**3.1.2 Characterization of bacterial cultures**

**3.1.2.1 Morphological identification**

Gram's staining was done for the morphological identification of the bacterial cultures. Our results showed that all the bacterial cultures were gram negative and were short rods similar to that of *Agrobacterium tumefaciens*.

**3.1.2.2 Colony characterization**

Colony characteristics of pure colony were used to identify bacterial cultures. The colony characteristics like colour, texture and margins were observed on YEM media. Colonies were white to creamish in colour and the texture showed that most of the colonies were smooth, mucoid, translucent and had glossy appearance. Most of the cultures had characteristics similar to that of *Agrobacterium tumefaciens*.

***3.2 Screening for CoQ10 potential***

The selection of CoQ10 producing bacterial culture was carried out based on the ability of bacterial cultures to grow on the selective medium plate. Culture S3, S6, G6, G9 and G12 were able to grow on the selective medium plate indicating potential to produce CoQ10**.** Out of five isolates, G12 was selected because it has shown fastest growing colonies on the selective media as compared to other isolates and hence used for further studies.

***3.3 Improvement by Random Mutagenesis***

**3.3.1 Effect of UV mutation on *Agrobacterium tumefaciens***

To enhance the production of CoQ10, native strain was subjected to UV mutation**.** The native strain showed reduction in number of colonies when exposed to mutagenic UV rays for 7 min**.** Isolate and its mutant were compared with A**(**609**) (***Agrobacterium tumefaciens* culture**)** and its mutant**.** Viability of cells reduced in both the cases**.**

**3.3.2 Effect of EMS mutation on *Agrobacterium tumefaciens***

To enhance the production of CoQ10, native strain was subjected to EMS mutation**.** The native strain showed reduction in number of colonies when treated with 80µM EMS for 30min**.** Isolate and its mutant were compared with A **(**609**), (***Agrobacterium tumefaciens* culture**)** and its mutant**.** Viability of cells reduced in both the cases**.** In case of EMS mutagenesis there was less reduction in viability as compared to UV mutagenesis**.**

***3.4 Screening of high CoQ10 producing mutant***

The selection of mutant was carried out based on the ability of mutants to grow on the selective media**.** G12 UV mutant has shown fastest growing colonies on the selective media indicating high potential to produce CoQ10 and hence it was taken for further studies**.**

**3.5 Spectrophotometer analysis**

The Quantitative analysis of CoQ10 content was done using spectrophotometer. Isolate G12 and its mutant were compared with A (609) and its mutant. Both the mutants showed increase in CoQ10 content. According to our results, CoQ10 content produced by G12 was 2.36 mg/ g DCW and G12 mutant was 4.34 mg/ g DCW respectively (Table 2). G12 mutant was further chosen for optimization of fermentation conditions for coenzyme Q10 production.

**Table 2: Coenzyme Q10 content (mg/ g DCW)**

| S. No. | Culture | CoQ10 (mg/ g DCW) |
|---|---|---|
| 1 | A(609) | 3.11 ± 0.135 |
| 2 | A(609) MUTANT | 4.86 ± 0.246 |
| 3 | G12 | 2.36 ± 0.3 |
| 4 | G12 MUTANT | 4.34 ± 0.104 |

**** Data in mean of three replicate ± S.D.***

***3.6 Optimization of coenzyme Q10 production in shake flasks***

**3.6.1 Effect of pH on CoQ10 content after fermentation by *Agrobacterium tumefaciens***

Culture pH has a significant effect on CoQ10 content**.** Batch cultures were studied for effect of pH ranging from 6**.**0 to 8**.**0 on CoQ10 production**.** The CoQ10 content at pH 7**.**0 **(**4**.**73 mg/ g DCW**)** was much higher than at pH 6**.**0 **(**1**.**32 mg/ g DCW**)** and at pH 8**.**0 **(**2**.**33 mg/ g DCW**) (**Table 3**).** It decreased due to a lower dry cell weight at acidic pH and a lower CoQ10 content at alkaline pH**.**

**3.6.2 Effect of temperature on CoQ10 content**

Cultures were incubated at different temperatures to study its impact on CoQ10 production. The study indicated that CoQ10 content increased gradually from 25° C **(**1**.**45 mg/g DCW**)** to 32° C **(**4**.**49 mg/ g DCW**),** but it decreased significantly at 35° C **(**2**.**69 mg/ g DCW**)** due to changes in physiological conditions **(**Table 4**).** CoQ10 content was maximum at 32° C **(**4**.**49 mg/ g DCW**).**

**3.6.3 Effect of inoculum load on CoQ10 content**

Batch cultures were studied with inoculum load ranging from $10^4$ CFU/ml to $10^8$ CFU/ml**.** At $10^4$ CFU/ml and $10^6$ CFU/ml of inoculum load the CoQ10 content was 1.24 mg/g DCW and 2.16 mg/ g DCW respectively. The maximum production of CoQ10 (4.55 mg/g DCW) was observed at $10^8$ CFU/ml of inoculum load (Table 5). The CoQ10 content increased with the increase in Inoculum load.

**Table 3. Effect of pH on CoQ10 content produced by G12 MUTANT**

| Coenzyme Q10 content (mg/ g DCW) | | |
|---|---|---|
| **S.No.** | **pH** | **G12 MUTANT** |
| 1 | 6.0 | 1.32 ± 0.199 |
| 2 | 7.0 | 4.73 ± 0.408 |
| 3 | 8.0 | 2.33 ± 0.405 |

**** Data in mean of three replicate ± S.D.***

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**Table 4: Effect of temperature on CoQ10 content produced by G12 MUTANT**

| Coenzyme Q10 content (mg/ g DCW) | | |
|---|---|---|
| **S. No.** | **Temperature** | **G12 MUTANT** |
| 1 | 25°C | 1.45 ± 0.336 |
| 2 | 32°C | 4.49 ± 0.533 |
| 3 | 35°C | 2.69 ± 0.273 |

**** Data in mean of three replicate ± S.D.***

**Table 5: Effect of inoculum load on CoQ10 content produced by G12 MUTANT**

| Coenzyme Q10 content (mg/ g DCW) | | |
|---|---|---|
| **S.No.** | **Inoculum load** | **G12 MUTANT** |
| 1 | $10^4$ CFU/ml | 1.24 ± 0.233 |
| 2 | $10^6$ CFU/ml | 2.16 ± 0.485 |
| 3 | $10^8$ CFU/ml | 4.55 ± 0.195 |

**** Data in mean of three replicate ± S.D.***

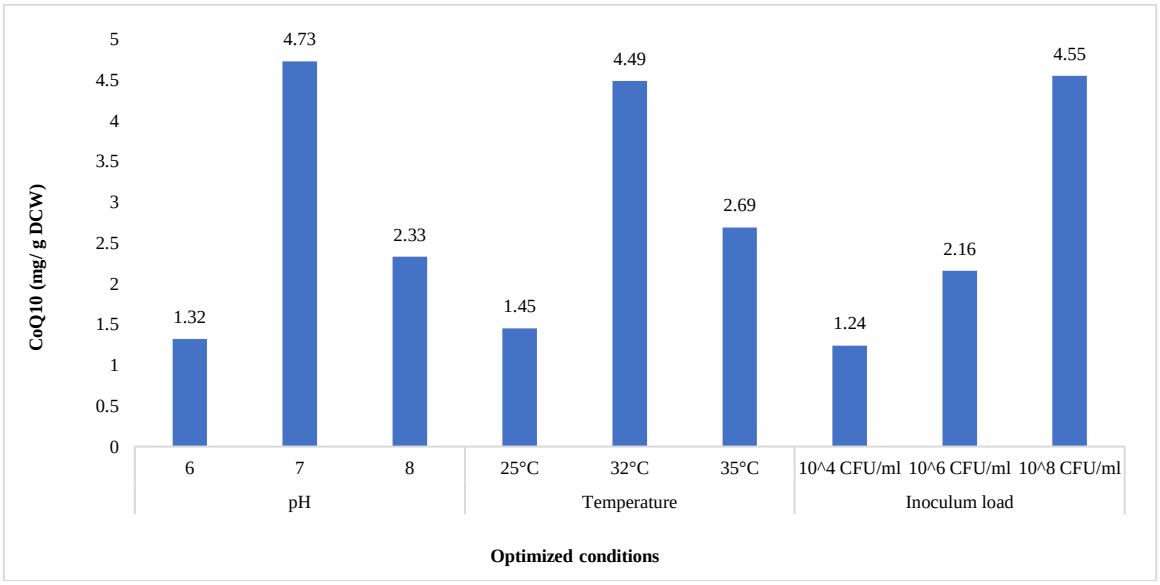


**Fig 1. Effect of various parameters on CoQ10 production, including pH, temperature, and inoculum load**

## IV. DISCUSSION

In present study, soil and gall samples were collected from vegetable field of Research farm located at Chatha, Jammu. Repeated isolations and purification resulted in 05 *Agrobacterium tumefaciens* cultures from soil samples and 07 from that of gall samples. Identification of cultures was confirmed by morphological studies (Bergey's Manual of Determinative Bacteriology). *Agrobacterium tumefaciens* is gram negative bacteria (Nester, 2015) commonly infecting dicotyledonous plants, both herbaceous and woody, (Rhouma *et al.*, 2006). Isolation of bacteria was done using Yeast Extract Mannitol agar and MacConkey selection media. MacConkey media has previously been utilised to isolate *Agrobacterium tumefaciens* from crown gall samples as a selective media. *Agrobacterium tumefaciens* colonies appeared white to creamish on YEM media (Shams *et al.*, 2012) and brick red on MacConkey selection media (Ali *et al.*, 2016). Bacterial isolates in our study were morphologically similar to that of *Agrobacterium tumefaciens.*

*Agrobacterium tumefaciens* cultures were screened on the selective media to select CoQ10 producing strains. Similar studies were also done by Yuan *et al.* (2012). G12 was best amongst 12 isolates studied and was chosen for further research on CoQ10 production potential. CoQ10 content produced by G12 was 2.36 mg/g DCW. Isolate G12 was further subjected to mutagenesis to enhance the CoQ10 content.

Random mutagenesis is a simple approach for making genetic and functional changes to an organism that leads to increased product yield, demonstrated using progressive stepwise mutagenesis-selection techniques and several mutagens with different modes of action (Chandra *et al.*, 2009). Mutagenesis using UV and EMS treatment was done to obtain a high-CoQ10-producing strain from the native isolate (G12). When exposed for 7 minutes to mutagenic UV radiation from a distance of 10 cm, the native strain (G12) showed loss in viability. Similar reports have been given by Tokdar *et al.*, (2013), who also reported loss of viability of cells when exposed to UV radiations. Bacterial colonies that survived UV exposure deemed to be mutant of G12 has shown increased potential to produce CoQ10. The results showed that the strain requires a longer exposure for mutations. EMS is a potent mutagen that causes point mutations in the DNA (Ranadive *et al.*, 2011) and in case of EMS mutagenesis, the viability of cells was reduced when they were treated with 80 µM EMS for 30 min but it was less effective than UV mutagenesis. After mutagenic treatment, screening was done to obtain high CoQ10 producing strain.

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Two methods of increasing CoQ10 production are possible, according to the general mechanism of CoQ10 production, the mutant may be able to avoid growth inhibition during CoQ10 biosynthesis, or its associated metabolisms may produce CoQ10 in excessive amount (Choi *et al.*, 2005). CoQ10 is an electron carrier in the respiration chain with antioxidant activity (Kawamukai, 2002). Sodium azide, an electron flow inhibitor is used to screen the mutant, that could be resistant to this inhibitor because of high intracellular CoQ10 levels (Choi *et al.*, 2005). In our studies, G12 UV mutant strain was selected after screening and tested for its CoQ10 production potential by flask culturing. Our results indicate that CoQ10 content increased from 2.36 mg/g DCW to 4.34 mg/ g DCW after mutagenesis, indicating positive mutations. G12 UV mutant was further used for optimization of fermentation conditions.

Batch cultures were studied with different inoculum loads at various temperatures and range of pH, to maximize CoQ10 production in 250ml flasks containing 100ml fermentation media along with constant parameters like agitation (180 rpm) and incubation time (96 hours). For optimization of pH, batch fermentation was performed at pH range 6.0 to 8.0, keeping other parameters constant. CoQ10 concentration at pH 7.0 (4.73 mg/ g DCW) was higher than that at pH 6.0 (1.32 mg/ g DCW) and at pH 8.0 (2.33 mg/ g DCW). It decreased due to a lower dry cell weight at acidic pH and a low CoQ10 content was reported at alkaline pH. Culture pH has a significant effect on CoQ10 content as compared to temperature (Ha *et al.*, 2007). During optimization of temperature, variable temperatures like 25˚C, 32˚C and 35˚C was used with other parameters being constant. It was observed that at 25°C, fermentation resulted in a decrease in CoQ10 content (1.45 mg/g DCW); hence 25°C may not be suitable for optimum growth and efficient utilization of substrates. Increase in temperature from 25°C to 32°C showed gradual increase in CoQ10 content but beyond that temperature, CoQ10 content decreased significantly at 35° C (2.69 mg/g DCW). The optimum temperature for CoQ10 production in 250 ml flask was 32°C with yield of 4.49 mg/g DCW. These results are consistent with the findings of Ha *et al.*, (2007b). In our study we also observed that maximum CoQ10 production occurs at inoculum load of $10^8$ CFU/ml (4.55 mg/g DCW). The CoQ10 content increased with the increase in Inoculum load. At $10^4$ CFU/ml and $10^6$ CFU/ml of inoculum load the CoQ10 content was 1.24 mg/g DCW and 2.16 mg/ g DCW respectively. The reason for this increase from $10^4$CFU/ml to $10^8$ CFU/ml could be due to the presence of more cells to perform fermentation.

Therefore, it was noted from our above study that amongst the culture conditions tested so far for CoQ10 production, G12 mutant strain showed maximum CoQ10 content when $10^8$ CFU/ml of inoculum load is used at 32°C and pH 7.0.

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