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6.1 INTRODUCTION
The downstream processing of secondary metabolites and other pharmaceutical important products from microbes vary with the nature of bacteria used, the quality and quantity of metabolite present in the fermented broth/biomass, viscosity and pH of the broth [1]. It has been noticed that the cost of downstream process is enormously higher as compared to the upstream process [2,3,4]. So, while developing a downstream process for isolation and purification of a biological product the number of steps is to be minimized. In the present work, a suitable microbial process by using a mutant strain of Paracoccus denitrificans was developed for the production of Coenzyme Q10 (C_{59}H_{90}O_{4}). CoQ10 having molecular weight 863.34. The product is intracellular available inside the bacterial cells. Hence, effective downstream process was developed in order to obtain the product at highest purity form and maximum quantity [5].

6.2 MATERIALS AND METHODS

6.2.1 Chemicals and Solvents
Sodium hydroxide and sulphuric acid (SRL, Mumbai-India)) was used for maintenance of broth pH. All solvents and reagents used for microbial culture, extraction, separation, product isolation, identification and purification were obtained from Qualigens (Mumbai, India). Coenzyme Q10 was purchased from Sigma Aldrich.

6.2.2 Microorganism and Fermentation conditions
A new strain of Paracoccus denitrificans was developed and selected by mutagenesis in author’s laboratory. The culture conditions were for the growth of Paracoccus denitrificans at shake flask level carried out as explained below. One loop of bacterial cells grown 48 h at 30°C on agar slant containing 0.5% yeast extract, 1.0% peptone, 0.5% NaCl and 2% agar (pH7.0) was inoculated to a 500 mL flask containing 100 ml of production medium composed of sucrose 20g l\(^{-1}\), ammonium chloride 1g l\(^{-1}\), potassium dihydrogen phosphate 2.3g l\(^{-1}\), di-sodium hydrogen phosphate 2.9g l\(^{-1}\), magnesium sulphate 0.5g l\(^{-1}\), sodium bi carbonate 0.5g l\(^{-1}\), calcium chloride 0.01 g l\(^{-1}\), ferrous ammonium citrate 0.05g l\(^{-1}\) (autoclaved separately), sodium succinate 6.75g l\(^{-1}\)
1 \textsuperscript{1}, yeast extract 10g l\textsuperscript{-1} and 1 ml l\textsuperscript{-1} trace elements solution [6]. The medium was sterilized at 121°C for 20 min. All components were purchased from Qualigens (Mumbai, India). The flasks were incubated for 48 h on a rotary shaker (Orbitek, SCIGENICS BIOTECH) at 250 rpm and 28°C. 60 L and 150 L liters fermentors were used for large scale culture. The conditions for 60 L and 150 L fermentors operation were as mentioned in chapter-2 and chapter-4. After completion of 72 hr the bacterial cells were harvested. Crude extract was prepared in n-propanol and n-hexane. The extraction process was optimized as described in result and discussion. 1\textsuperscript{st} and 2\textsuperscript{nd} Extracted organic phase was combined and concentrated to dryness. This extract was redissolved in small amount of n-hexane and was purified through silica gel adsorption chromatography and subsequent crystallization.

6.2.3 Equipments and Analytical methods
A glass column (440 mm $\times$ 25 mm id) was purchased from borosil glass. The adsorbents used for column chromatography was analytical grade silica gel (60-300 mesh). About 70 gm silica was mixed in hexane to make 1 gm/ml sample slurry solution, which was then loaded on the top of the silica gel column. Various solvent systems were tried in combination and alone for CoQ10 elution among them hexane and dichloromethane shows best results, so the solvent system used for column chromatography was Hexane: Dichloromethane (2:1).The column was eluted at a flow rate of about 2 ml/min and the effluent was collected in each 10 ml fractions. The orange yellow fractions containing CoQ10 were analyzed by HPLC and the fractions with the HPLC purity above than 95% was combined and concentrated. The purified CoQ10 was redissolved in small amount of ethyl alcohol (Ethanol) and crystallized by gradually cooling down to 4°C and stored overnight. Crude extract and each fraction were analyzed using a High Performance Liquid Chromatography (Shimadzu, Model LC20, Class VP). HPLC specification was as column C-18 (150 mm $\times$ 4.6 mm i.d.5 $\mu$m), column oven temperature 25°C. The mobile phase consist of methanol: ethanol (80:20 v/v with 5.6 gm/L sodium perchlorate) was isocratically eluted at a flow rate of 1 ml/min. Detector wave length 275 nm on UV-Vis detector.
6.3 RESULTS AND DISCUSSIONS

6.3.1 Effect of various solvents on CoQ10 extraction from broth

The broth obtained after fermentation process was having intracellular CoQ10 and its extraction process optimization was carried out to transfer maximum product in solvent phase from the cell biomass. [2, 7, 8, 9]. At this stage the pH of the broth was 7.4-7.8. Various polar and non-polar solvents available at commercial level were tested to recover CoQ10 from aqueous phase. Figure-1 shows that the hexane is the best solvent for the extraction of CoQ10 from lysozyme disrupted cell suspension as compared to other solvents.

![Fig 1](image)

**Figure 1 Effect of various solvents on extractability of CoQ10 (after cell lysis)**

Among the various solvent tested, hexane was noticed to be the most effective solvent. The ratio of cell suspension and solvent mixture (Hexane and propanol) was also optimized and noticed to be 1:1 (Figure 2). The further optimization of solvent system was n-hexane and n-propanol in 2:1 and broth solvent ratio was optimized (1:2) (Figure 3 and 4) respectively.
Figure 2 CoQ10 Extraction efficiency of different Solvent systems in combination with n-hexane

Figure 3 Optimization extraction ratio of best Solvent system n-hexane and n-propanol
pH also play an important role in extraction process and stability of the product. So role of pH in extraction process is studied and found that neutral pH i.e. 7-8 is the better for the extraction of CoQ10 and give maximum yield (Figure-6).
Triton X 114, NaCl, and SDS (sodium dodecyl sulphate) found to be enhancer in cell disruption so they are also used in extraction process. The quantities of these components are mentioned in materials and methods.

The mixture of broth and solvent mixture was kept on shaking for 2 h at 32-35°C. With a gap of 30 minutes the organic layer were tested for CoQ10. It was noticed that a period of 2h extraction was sufficient for maximum extraction of CoQ10 from broth and the same was tested by HPLC (Figure-8).
Figure 8 Effect of time duration on CoQ10 extraction

About 95% CoQ10 and their derivatives were recovered. After the completion of extraction process the organic layer was separated from the aqueous phase. The residual CoQ10 was separated from the aqueous phase. The process of evaporation was carried out at ambient temperature till the final extract volume was reduced to 10%. The concentrated extract was used for recovery and purification of the final product by column chromatography.

Figure 9 Purification of CoQ10 through column chromatography
Flow Diagram of isolation and Purification process of Coenzyme Q10 from Fermentation Broth

1st Extractor
Solvent: Cell Suspension (2:1)

2nd Extractor
Solvent: Cell suspension (1:1)

3rd Extractor
Solvent: Cell suspension (1:1) (optional)

Concentrated Extract

Concentration of pooled Extract

Column Chromatography

Crystallization

Crystal powder
Besides this, FDA has strictly banned on any residual contamination of hexane or hexane like solvents in the finished product. In order to avoid such problems due care has been taken in purification process. The crude extract was subjected to vacuum evaporation in order to minimize its quantity [9,10]

**6.3.3 Isolation and Purification of CoQ10 by column chromatography**

The solvent system used for column chromatography was Hexane: Dichloromethane (2:1). The column was eluted at a flow rate of about 2 ml/min and the eluent was collected in each 10 ml fractions. The orange yellow fractions containing CoQ10 were analyzed by HPLC and the fractions with the HPLC purity above than 95% was combined and concentrated. The purified CoQ10 was redissolved in small amount of Ethyl alcohol, and crystallized by gradually cooling down to 4°C and stored overnight. Crude extract and each fraction were analysed using a High Performance Liquid Chromatography (Shimadzu, Model LC20, Class VP). HPLC specification was as column C-18(150 mm x 4.6 mm i.d.5 μm), column oven temperature 25°C. The mobile phase consist of methanol: ethanol (80:20 v/v and 5.6 g/l sodium perchlorate) was isocratically eluted at a flow rate of 1 ml/min. detector wave length 275 nm on UV-Vis detector. The HPLC chromatograms are shown in “Annexure IV”.

**6.3.4 Crystallization process**

This mixture of elute and ethanol was subjected to 4°C temperature for a period of 18h in order to obtain crystallized CoQ10 [11,12,13,14]. The supernatant layer was separated from the crystal by filtration and the semi-dried CoQ10 was kept in a vacuum oven at 48 °C for a period of 3h. The crystallized product was tested for purity by HPLC (Annexure IV). The purity of the final product was noticed to be 99%. About 75% recovery was obtained by this optimized downstream process. The absorbance spectrum was checked of this Coenzyme Q10 in spectrophotometer (Hitachi) (Figure 10).
Nitrogen gas/ 0.5 Kg pressure
Concentrated Extract (Solvent + CoQ10)

COLUMN
PURIFICATION
AND
CRYSTALLIZATION

Packing of CoQ10 powder

Nitrogen gas/ 0.5 Kg pressure
Concentrated Extract (Solvent + CoQ10)

COLUMN
PURIFICATION
AND
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Packing of CoQ10 powder

Nitrogen gas/ 0.5 Kg pressure
Concentrated Extract (Solvent + CoQ10)

COLUMN
PURIFICATION
AND
CRYSTALLIZATION

Packing of CoQ10 powder

Ethyl Alcohol

Purified Product

Crystallizer

Coenzyme Q10

Spent Solvent Mixture

Recycle
Figure 10 Maximum absorbance spectra of CoenzymeQ10
6.4 REFERENCES


