Strain improvement of *Sporidiobolus johnsonii* -ATCC 20490 for biotechnological production of Coenzyme Q_{10}

Prafull Ranadive, Alka Mehta, and Saji George

Abstract—The strain improvement of *Sporidiobolus johnsonii* ATCC 20490 for improved production of Coenzyme Q_{10} (CoQ_{10}) was undertaken. Ultraviolet (UV) rays and Ethyl Methane Sulphonate (EMS) were used as a mutagenic agent to generate mutant strains, out of which the desired mutants having resistance to high temperature and various inhibitors like atorvastatin, ethionine, capsaicin, were rationally selected. Screening of the selected desired mutants were carried out by submerged cultivation followed by extraction and quantification of CoQ_{10}. Atorvastatin resistance marker was found to be a useful tool in selecting high CoQ_{10} producing mutants. EMS induced atorvastatin resistant mutant strain EA22 generated from *Sporidiobolus johnsonii* ATCC 20490 showed two-fold increase in CoQ_{10} titer. The mutant strain was morphologically different from parent strain in terms of colony size, pigmentation on agar and showed marked differences in the shape and size of yeast cells as seen from Scanning Electron Microscopic (SEM) observation, indicating phenotypic changes.

Index Terms—*Sporidiobolus johnsonii*, *Coenzyme Q_{10}*, Mutant, SEM, EMS, UV.

I. INTRODUCTION

Coenzyme Q_{10} (CoQ_{10}) an isoprenylated benzoquinone, is a well-known component of the electron-transfer system in eukaryotes. CoQ_{10} primarily functions as an electron transporter in aerobic respiration and oxidative phosphorylation in the respiratory chain located in the mitochondrial inner membrane of eukaryotes. It also functions as a lipid soluble antioxidant in cellular biomembranes that scavenges reactive oxygen species [1]. Nowadays CoQ_{10} is widely used as an oral nutritional supplement and is beneficial in the treatment of several human disorders like cardiomyopathy, diabetes and neurodegenerative diseases and also diseases associated with CoQ_{10} deficiency.

Due to increased demand for CoQ_{10} as an active ingredient in formulations, several processes have been developed for its commercial production. The economical production of CoQ_{10} using biological processes is becoming more widespread. The biological process predominantly depends on microbes that produce high levels of CoQ_{10} naturally. CoQ_{10} containing organisms including *Agrobacterium tumefaciens*, *Paracoccus dentrificans*, *Cryptococcus laurentii*, *Tricosporon sp.*, *Sporobolomyces salmonicolor* & *Rhodobacter sphaeroides* have been used to optimize fermentation process for the production of CoQ_{10}. The improvements in CoQ_{10} titer have been achieved using mutant strains with several fold increase in titers [2].

The biosynthetic pathway of CoQ_{10} in microorganisms has been reported. The metabolic and genetic engineering of microorganisms targeting specific biochemical pathway has been reported for production of CoQ_{10} but the production levels are not yet competitive with the levels presently produced by random mutagenesis and screening. The successful approaches for the commercial production of CoQ_{10} have relied predominantly on bacterial and yeast mutants selected for their high CoQ_{10} content. So far, the isolation of strains by mutagenesis and selection on inhibitors has proved to be the most successful strategy to increase yields of CoQ_{10} [5, 7, 8].

Sporidiobolus johnsonii, a heterobasidiomycetes yeast strain is known to produce CoQ_{10} when cultivated under submerged conditions and there is only one report on its bioprocess [3]. The process of producing mutagenized *Sporidiobolus ruineniae* strain for CoQ_{10} production was described but no work has been reported on the yield improvement of CoQ_{10} in *Sporidiobolus johnsonii* [6]. Being a eukaryote, the naturally CoQ_{10} producing *Sporidiobolus* can be a suitable organism for CoQ_{10} bioprocess if the yields are promising. Although the natural yields are low, there is a scope to improve the yields by classical strain improvement [9, 10, 11]. The knowledge of the CoQ_{10} biosynthetic pathway may be useful for designing the rational strategy of mutant selection.

The objective of this study was to standardize the mutagenesis, selection and screening protocol in order to get higher CoQ_{10} producing mutant strain of *Sporidiobolus johnsonii* ATCC 20490. We demonstrate the rational approach of selecting mutants based on the resistant markers. The resistant markers were decided based on the tolerance of the strain to the oxidative stress and the inhibitors of the biosynthetic pathway of CoQ_{10}. It is hypothesized that the resistant mutants may overcome the inhibition by increased production of CoQ_{10}. The rationally selected strains were screened to obtain the desired higher CoQ_{10} producing strain. Morphological characterization of the best mutant was carried out to establish the difference from the parent strain.

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II. MATERIALS AND METHODS

A. Strains and Morphology

The heterobasidiomycetes yeast strain *Sporidiobolus johnsonii* ATCC 20490 and its induced mutants were maintained on the Yeast Malt Agar (YMA) slants. They were streaked on YMA plates and incubated at 30ºC for 4-5

days to study the colony morphology.

B. UV mutagenesis

The culture was grown overnight in 40ml of Yeast Malt Broth medium in 500 ml flask to get viable count of around 10^8cfu/ml. Five ml of the suspension was placed in sterile petri dish and exposed to UV rays (235nm) at a distance of 10cm. At regular intervals, the samples were taken out and different dilutions were plated on YMA to determine viable count. The percent kill rate was calculated by comparing viable count with that of unexposed suspension.

C. EMS mutagenesis

The culture was grown overnight in 40ml of Yeast Malt Broth medium in 500 ml flask to get viable count of around 10^8cfu/ml. The cells were centrifuged, washed and suspended in 10mM phosphate buffer (pH 7.0) to which EMS was added at the rate of 20μl/ml. The content was mixed and at regular intervals, 5ml of sample was taken out to which 5ml of 5% sodium thiosulfate was added to stop the mutagenesis. It was centrifuged to get pellet, reconstituted with 5ml buffer and different dilutions were plated on YMA to determine viable count. The percent kill rate was calculated by comparing viable count with that of untreated suspension.

D. Rational selection of mutants

The atorvastatin, ethionine and capsaicin were tested in plate to find out its inhibitory effect on the yeast cells. The atorvastatin resistant mutants were selected by incorporating different concentrations of the drug in plate (20, 50, 100μg/ml). The mutant suspension was swabbed on to the plates and incubated at 30°C for 4-5 days for colonies to appear. The desired colonies were transferred to YMA slants.

To select the mutant resistant to high temperature, the kill curve of parent was determined by exposing cell suspension at 50ºC in water bath shaker. At regular intervals, the samples were taken out and different dilutions were plated on YMA to determine viable count. The percent kill rate was calculated by comparing viable count with that of untreated suspension. Appropriate exposure time was selected from kill curve and used to treat the mutegenized suspension at 50ºC and surviving mutants were selected.

E. Screening of mutants

The parent strain and selected mutants were inoculated in 5ml seed medium (Glucose 1%, Yeast extract 0.3%, Malt extract 0.3%, Peptone 0.5%, pH 6.0) in test tube and incubated at 30ºC with shaking at 220rpm. After 24h, 2.5% of the seed was transferred to 20ml production medium (Glucose 3%, Yeast extract 0.5%, Malt extract 0.5%, Peptone 1%, pH 6.0) in 100ml flask and incubated at 30ºC with shaking at 220rpm for 90 to 96h.

F. Extraction and quantification of CoQ10

The entire 20ml harvested broth was centrifuged at 1000rpm for 20min to get biomass pellet, which was extracted, with 20ml ethanol by heating in shaking water bath at 60°C for 3h. The cells were removed by centrifugation and ethanol layer was re-extracted with 20 ml hexane. The hexane layer was separated, concentrated to

1ml and 20μl was loaded to silica gel plate using win Cats based spotter for quantitative estimation by HPTLC. Mobile phase used was hexane: ethyl acetate (9:1). After separation, the plates were scanned using Camag TLC scanner to get the area under the curve for corresponding spots having identical retention time (RT) matching with standard CoQ_{10}. The titer was estimated by comparing the area of sample and standard of known concentration and expressed as mg of CoQ_{10} per liter of broth (mg/L).

G. Scanning Electron Microscopy (SEM)

The one-week-old growth of parent strain and mutant strain EA22 on the slants were scrapped to make water suspension, fixed with 2.5% glutaraldehyde and observed under FEI Quanta 200 Environmental scanning electron microscope at different magnifications.

III. RESULTS AND DISCUSSION

Strain improvement of CoQ_{10} producing bacterial and yeast strains have been reported, but this is the first report on *Sporidiobolus johnsonii* strain improvement. In the present study, *Sporidiobolus johnsonii* strain was subjected to genetic manipulation by physical and chemical mutagenesis to generate mutant strains followed by rational selection of mutants by exerting a selection pressure using various inhibitors. The conditions for mutagenesis were optimized based on the kill curve and the kill around 90% and above was considered as acceptable.

UV induced mutation showed around 90% kill with exposure at 20min and by 30min there was least survival, as shown in figure 1. It is clear from the results that the strain requires higher exposure time for kill and it may be due to the presence of resistant teliospores of the yeast. EMS is a potent mutagen that induces point mutation in the DNA. It was reported that EMS is useful mutagen in strain improvement of yeast cells. It greatly increased induction of auxotrophic mutants and also helped to improve the biomass yield and protein [4]. EMS treatment to *Sporidiobolus johnsonii* yeast cells at 20μg/ml showed gradual kill starting from 10min to 40min exposure time as shown in figure 2. The exposure for 30min was desirable to get mutants as it showed 92% kill. The UV and EMS induced mutagenic treatment were carried out on the parent yeast cell suspension so as to get above 90% kill rate generating mutagenised suspension.
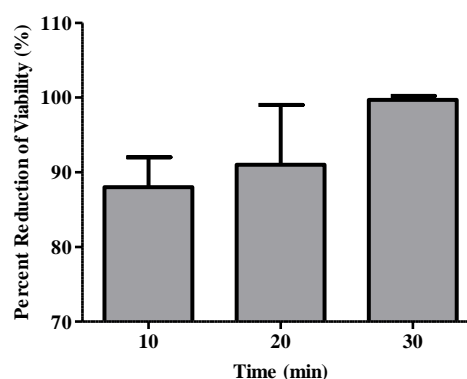

Figure 1. UV mutagenesis: Percent reduction of viability with time of UV exposure
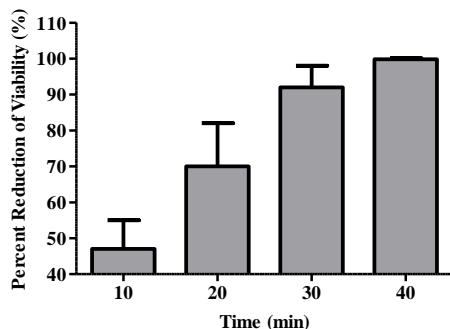
Figure 2. EMS mutagenesis: Percent reduction of viability with time of exposure to EMS (20 μl/ml)

Random selection and screening of mutants is a time consuming laborious process. We have adopted the rational selection approach based on the selection of mutants having resistance to high temperature and various inhibitors of the biosynthetic pathway like atorvastatin, ethionine, and capsaicin, to selectively enrich higher producer strains.

Heat stress is one of the causes for reactive oxygen species induced cell damage and the tolerance is gained by the regulation of intracellular redox state. Since CoQ_{10} is one of the main redox controllers of the cell, the heat resistance was considered as selecting criteria for CoQ_{10} overproducing mutants. Heat treatment was used to induce oxidative stress generating the free radicals and the resistant mutants were thought to survive in presence of oxidative stress through increased production of antioxidants, which may be CoQ_{10}. To select the temperature resistant mutants, the kill curve was obtained by exposing the parent cell suspension at 50 ℃ for different time period. Figure 3 shows the kill rate obtained by exposure at 50 ℃ at different time periods out of which 10min exposure was found to be appropriate for mutant selection as it showed 90% kill. The mutagenized suspension was exposed to these conditions and surviving temperature resistant mutants were selected on YMA plate.

Various inhibitors were tested for rational selection of mutants based on the CoQ_{10} biosynthetic pathway and properties. Atorvastatin, a competitive inhibitor of HMG-CoA reductase enzyme in CoQ_{10} biosynthetic pathway, Ethionine, an anti-metabolite of methionine which gives methyl group and Capsaicin, an inhibitor of plasma membrane redox system were used in the study. In the preliminary screening, the ethionine and capsaicin showed poor inhibition whereas atorvastatin showed clear inhibition with yeast strain as shown in figure 4(a). The minimum inhibitory concentration (MIC) of atorvastatin was estimated by incorporating different concentrations into the agar plate and it showed MIC value of 10μg /ml.
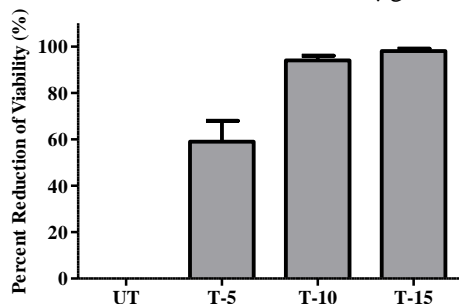

Figure 3. Effect of heat treatment (50 ℃) on percent reduction of viability with time of exposure
UT- Untreated, T-5 – 5min exposure, T-10 – 10min exposure, T-15 – 15min exposure.

The ethionine and capsaicin were not followed for mutant selection, as it did not show clear inhibition even at higher concentrations. Atorvastatin inhibition clearly indicated that the mevolonate pathway leading to CoQ_{10} biosynthesis exists in *Sporidiobolus*, similar to that of mammals, which is inhibited by cholesterol lowering statins drugs. Atorvastatin resistant mutants are thought to overcome the inhibition with increase in fernesyl pyrophosphate pool for CoQ_{10} biosynthesis. In prokaryote mainly non-mevalonate pathway called MEP pathway is reported [1].

Atorvastatin concentrations above MIC like 20μg/ml, 50 μg/ml and 100μg/ml were used to select the resistant mutants. The mutagenized suspension was swabbed onto these plates and the surviving resistant colonies were picked up for screening. Figure 4(b-2) shows the mutants obtained on atorvastatin plate and Figure 4(b-1) showing colonies of parent strain on YMA plate. It was observed that majority of atorvastatin resistant mutants were less pigmented than the parent colonies and showed marginal reduction in size of the colony. Mutants selected based on the temperature resistance did not show any change in colony shape and color.

The mutant selected by the above process were screened and compared for CoQ_{10} production. Figure 5 shows the TLC profile of some mutants and standard CoQ_{10} along with their area under the curve. The concentration of CoQ_{10} in the mutant strains were estimated and compared with parent strain, which was based on their respective AUC. Fifty mutants from each selection were screened.

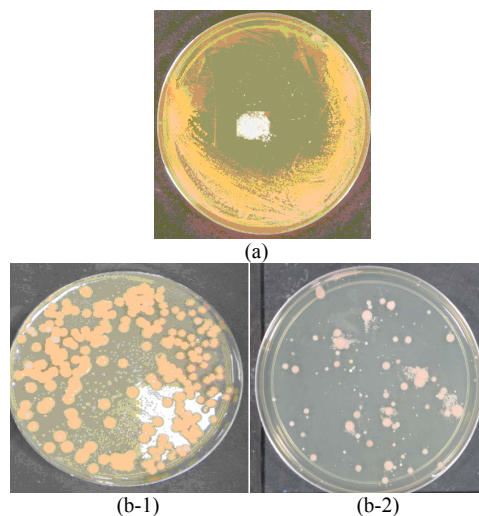(a)

(b-1)   (b-2)

Figure 4. (a). Inhibition due to Atorvastatin, (b-1). Parent colonies on YMA plate, (b-2). Atorvastatin resistant mutant colonies.

Overall from the screening results, 21% of atorvastatin resistant mutants showed improved CoQ_{10} as compared to parent. The temperature resistant mutants did not show any improvement in CoQ_{10} titer, and many of them showed reduction in titer. It was assumed that the temperature induced oxidatative stress might result in free radical generation to which the surviving resistant mutant may respond by overproduction of cellular antioxidants that may include CoQ_{10}. However this mechanism is not very specific to CoQ_{10} as there are several antioxidant proteins in the cell machinery. Out of fifty less pigmented atorvastatin (20 μg/ml) resistant mutants, an EMS induced mutant named EA22 showed highest titer which was seen as a dark spot of CoQ_{10} from the HPTLC profile as shown in Figure 5.

This mutant was tested along with parent strain in three consecutive shake flask runs which showed two fold increase in titer as shown in Figure 6.
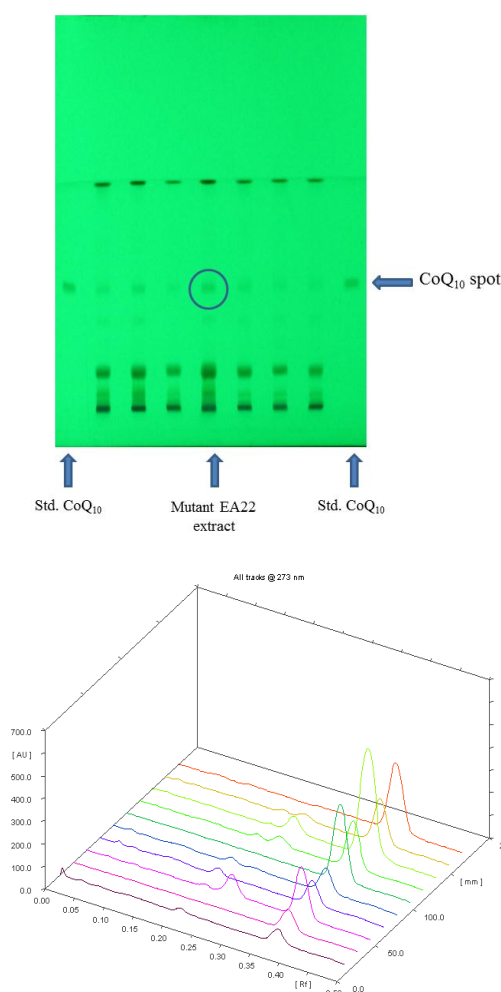

Figure 5. HPTLC profile of extracts from different mutant strains along with standard CoQ_{10}, showing area under curve (AUC) at bottom. Mutant EA22 showing higher yield seen as a dark spot of CoQ_{10}
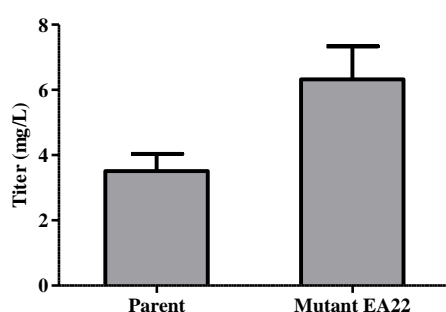

Figure 6. Comparison of CoQ_{10} titer between Parent and Mutant EA22: Average of three times testing

The mutant strain EA22 was morphologically different than parent hence scanning electron microscopy study was conducted for the parent and mutant strain to differentiate them on the basis of the shape and size of the yeast cells. The morphological characterizations of wild and mutant strains of *Candida tropicalis* using SEM have been reported in which the mutant strain was found to have numerous chlamydospores [4]. Figure 7 shows the morphology of parent and mutant strain under environmental Scanning Electron Microscope at 10,000x magnification, which showed the reduction in the shape and size of the mutant yeast cells. The parent yeast cells were elongated oval shaped measuring average length of 7-8 micron whereas the mutant cells were smaller and slightly oval having average length of 4-5 micron. The microscopic observation at higher magnification clearly indicated the change in morphology of mutant strain brought by induced mutation. Hence it may be necessary to evaluate the changes at genetic level using molecular techniques.
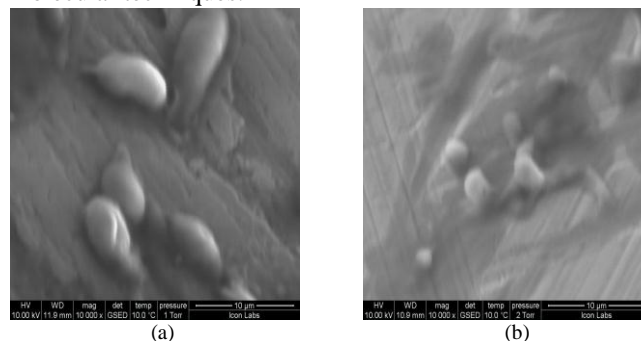

Figure 7. Scanning electron micrograph of yeast cells: (a) parent and (b) mutant EA22 showing the reduction in size and difference in shape

IV. CONCLUSIONS

Rational selection of induced mutants of *Sporidiobolus johnsonii* based on the atorvastatin resistance increased the chance of selecting higher CoQ_{10} producing mutants. From this selection, an EMS induced mutant strain EA22 showed two-fold increase in titer than parent, which was highest among all mutants. The rise in titer was reproducible indicating the stable characteristics of the mutant. It is possible that atorvastatin resistance may upregularte the genes responsible for HMG CoA reductase enzyme to which it binds thereby increasing the metabolite pool in the biosynthetic pathway of CoQ_{10}. The gene expression studies can prove this hypothesis. The mutations have brought phenotypic changes as seen with the mutant strain EA22. The mutant colony is less pigmented than the parent, indicating occurrence of some mutation at pigment biosynthesis step. There is a possibility that the isoprene precursors of the pigment production might have been diverted towards CoQ_{10} production. The genotypic changes in the mutant can be further evaluated using molecular techniques. Sequential re-mutagenesis and screening process on this mutant is proposed to generate a mutant strain with several fold increase in titer.

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