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ORIGINAL ARTICLE

Morphological and Molecular Differentiation of *Sporidiobolus johnsonii* ATCC 20490 and Its Coenzyme Q_{10} Overproducing Mutant Strain UF16

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Abstract Coenzyme Q_{10} (CoQ_{10}) is an industrially important molecule having nutraceutical and cosmeceutical applications. CoQ_{10} is mainly produced by microbial fermentation and the process demands the use of strains with high productivity and yields of CoQ_{10}. During strain improvement program consisting of sequential induced mutagenesis, rational selection and screening process, a mutant strain UF16 was generated from *Sporidiobolus johnsonii* ATCC 20490 with 2.3-fold improvements in CoQ_{10} content. EMS and UV rays were used as mutagenic agents for generating UF16 and it was rationally selected based on atorvastatin resistance as well as survival at free radicals exposure. We investigated the genotypic and phenotypic changes in UF16 in order to differentiate it from wild type strain. Morphologically it was distinct due to reduced pigmentation of colony, reduced cell size and significant reduction in mycelial growth forms with abundance of yeast forms. At molecular level, UF16 was differentiated based on PCR fingerprinting method of RAPD as well as large and small-subunit rRNA gene sequences. Rapid molecular technique of RAPD analysis using six primers showed 34 % polymorphic fragments with mean genetic distance of 0.235. The partial sequences of rRNA-gene revealed few mutation sites on nucleotide base pairs. However, the mutations detected on rRNA gene of UF16 were less than 1 % of total base pairs and its sequence showed 99 % homology with the wild type strain. These mutations in UF16 could not be linked to phenotypic or genotypic changes on CoQ_{10} biosynthetic pathway that resulted in improved yield. Hence, investigating the mutations responsible for deregulation of CoQ_{10} pathway is essential to understand the cause of overproduction in UF16. Phylogenetic analysis based on RAPD bands and rRNA gene sequences coupled with morphological variations, exhibited the novelty of mutant UF16 having potential for improved CoQ_{10} production.

Keywords *Sporidiobolus johnsonii* · Coenzyme Q_{10} · Mutant · RAPD · Strain improvement · Atorvastatin · Free radical · Mutation

Introduction

Coenzyme Q_{10} (CoQ_{10}) is commercially important nutraceutical compound used extensively in supportive treatment of cardiomyopathy, cancer, neurodegenerative diseases and diseases associated with CoQ_{10} deficiency [1, 2]. Structurally, it is a benzoquinone with chain of ten isoprene units attached to it. It functions as an electron transport agent in respiratory chain generating ATP and a potent lipid soluble antioxidant preventing lipid peroxidation [3]. CoQ_{10} level in the body is a measure of health since the low levels are associated with ageing and related diseases causing decreased energy and susceptibility to oxidative stress. The

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oral formulations containing CoQ_{10} have proved to be useful in improving serum CoQ_{10} levels and its cosmetic formulations has an anti-ageing property; therefore there is great demand for this molecule in the market [1, 4].

Production of CoQ_{10} using chemical synthesis has several disadvantages in terms of purity, cost and selectivity, hence microbial fermentation processes are widespread and scores over synthetic route of CoQ_{10} production [5]. The selection of suitable strains and further improving the productivity is prerequisite for cost effective production of CoQ_{10} by fermentation [1]. Therefore research efforts on the production of CoQ_{10} by microorganisms are focused on the development of potent strains by conventional mutagenesis and metabolic engineering, analysis and modification of the key metabolic pathway, and optimization of fermentation strategies [4]. The *E. coli* produces CoQ_8 whereas *S. cerevisiae* produces CoQ_6; therefore these well established strains are not suitable for CoQ_{10} fermentation. The genetic engineering efforts for CoQ_{10} production in *E. coli* and *S. cerevisiae* did not give very encouraging results in terms of production yields [1, 6–9]. Hence, the commercial process relies on bacterial and yeast strains, which are natural producers of CoQ_{10}. Improving strains by mutagenesis followed by selection on inhibitors, is a successful strategy to increase the yields of CoQ_{10} [1, 8, 10]. Mutagenesis process using low-energy ion beam irradiation [11], menadione [12], high hydrostatic pressure treatment, UV and diethyl sulfate [13] have been reported for CoQ_{10} strain improvement of bacterial strains. For rational selection of CoQ_{10} overproducing bacterial mutants, the inhibitors like L-ethionine, daunomycin, menadione, vitamin K3 and sodium azide have been used [13, 14]. These induced mutants showed improvements in CoQ_{10} yield, but there are no reports on molecular characterization of these mutants to differentiate it from the original parent strains.

Sporidiobolus johnsonii, a heterobasidiomycetes yeast strain was known to produce CoQ_{10} under submerged cultivation [15]. The isolation of CoQ_{10} from *S. johnsonii* has been reported by one group, using improved fermentation process involving feeding of *para*-hydroxy benzoic acid, a precursor of benzoquinone moiety of CoQ_{10} [16]. This strain has not been explored extensively for CoQ_{10} studies like few higher producing bacterial strains *Agrobacterium tumifaciens* (*Rhizobium radiobacter*) and *Rhodobacter sphaeroides* [2] due to its low CoQ_{10} content, hence the strain improvement work on this strain was undertaken by us [17]. Improving the yields of CoQ_{10} in *S. johnsonii* would be beneficial so as to take the advantage of having more biomass production in yeast fermentation. Additionally, yeasts like *S. johnsonii* becomes a promising source of natural CoQ_{10} derived from eukaryote kingdom having high degree of conservation with mammalian system and its biosynthetic pathways [18]. During strain improvement program on this strain, the improved mutant strain EA22 was previously generated by EMS mutagenesis followed by rational selection [19] on atorvastatin (20 μg ml^{-1}) [17]. Re-mutagenesis process brings about improvement in yields [20]; hence we continued our efforts on this strain to bring significant improvement in CoQ_{10} content by induced re-mutagenesis and rational selection.

The strains that are developed by induced mutations are genotypically and or phenotypically different from their respective parent strains [21, 22], hence investigation of these changes are essential to characterize them as a novel strains. Morphological variations and molecular differentiation methods like rRNA gene sequences, RAPD, AFLP or RFLP are being used to discriminate the strains of same genera or species and mutants [21, 23–26]. The objective of this study was to generate the mutant strain of *S. johnsonii* having significant improvement in CoQ_{10} content and to differentiate it from wild type strain, based on cultural and microscopic morphology, rRNA gene sequences and physiological attributes. Efforts were made to develop a molecular method based on RAPD for rapid differentiation of mutant strain.

Materials and Methods

Strains, Media and Materials

The yeast strain *S. johnsonii* ATCC 20490 and its generated induced mutants were maintained at 4–8 °C on yeast malt (YM) agar slants (glucose 10 g l^{-1}, peptone 5 g l^{-1}, yeast extract 3 g l^{-1}, malt extract 3 g l^{-1}, agar 20 g l^{-1}, pH 6.0). They were streaked on YM agar plates and incubated at 28–30 °C for 4–5 days to study the colony morphology. All dehydrated media and media components were procured from Hi-Media, India. All solvents (AR grade) were procured from Merck.

Mutagenesis and Selection of Mutants

Mutant EA22 that was previously generated from wild type strain *S. johnsonii* ATCC 20490 [17] was used as a starting strain for further improvement. Natural variant of this mutant, E2A22 was selected on YM agar plate, tested in shake flask and further re-mutated using combined exposure of UV and free radicals. The culture was grown for 16 h at 28–30 °C on shaker with 220 rpm in 500 ml flask containing 40 ml of Yeast Malt (YM) broth medium, to get log phase growth of cells having viable count of around 10^8 cfu ml^{-1}. The cells were centrifuged, washed and suspended in 10 mM phosphate buffer (pH 7.0). The 10 ml suspension was added to sterile petri plates (90 mm size) to which filter sterilized stock solutions of riboflavin and

methionine were added to get the final concentration of 2 ppm and 6,000 ppm respectively [27]. The contents were mixed and exposed to UV rays (254 nm) at a distance of 10 cm. Another set of suspension without riboflavin and methionine was exposed to UV rays which served as a control. At regular intervals, the samples were taken out and kept in the dark for at least 2 h to prevent the light dependent DNA repair mechanism followed by plating on YM agar plates to determine viable count. The samples were simultaneously plated on separate YM agar plates containing 20 and 40 μg ml^{-1} of atorvastatin (Biocon Limited, India) respectively, for selection of resistant mutants. The viable count was determined from the treated and untreated suspension in order to get the survival count. The mutant colonies on atorvastatin containing plates were purified on YM agar plates without atorvastatin and transferred to plain YM agar slants.

Shake Flask Fermentation

The seed medium (YM Broth) contained (g l^{-1}): glucose 10, yeast extract 3, malt extract 3, peptone 5, pH 6.0. The 40 ml of seed medium dispensed in 500 ml flask was inoculated with stock cultures from 2 to 4 weeks old YM slants and incubated at 28–30 °C on rotary shaker having 220 rpm, for 24 h. The production medium (YMP) used for screening of mutants in shake flask contained (g l^{-1}): glucose 30, yeast extract 5, malt extract 5, peptone 10, pH 6.0. The 50 ml of medium dispensed in 500 ml flask was inoculated with 10 % seed and incubated at 28–30 °C on rotary shaker having 260 rpm. The flasks were harvested at 90–96 h. During the screening of mutants, the respective parent strains were included along with mutant strains in shake flask fermentation which served as a control.

Measurement of Dry Cell Weight (DCW)

The 20 ml of broth sample was centrifuged at 6,000 rpm for 15 min. The pellet was washed with water and lyophilized. Lyophilized biomass was weighed to obtain dry cell weight (DCW) and expressed in g l^{-1}.

Extraction of CoQ_{10}

The modification of the reported method [15] was used for extraction of CoQ_{10}. 10 ml broth sample was centrifuged at 12,000 rpm for 20 min. The resulting wet biomass was freeze thawed and mixed with 20 ml ethanol in 100 ml flasks with secured lid. The mixture was sonicated and transferred to a shaker water bath at 60 °C with constant stirring for 2 h. After 2 h of heating, 20 ml n-hexane was added to it and placed in shaker water bath at 60 °C for further 1 h. The solution was centrifuged at 6,000 rpm for 20 min to remove the ruptured cell debris. The solvent layer was separated and 5 ml distilled water was added to it to get the layer separation. The top Hexane layer was separated. The spent ethanol–water layer was re-extracted with n-hexane. The spent biomass was subjected to re-extraction by above method and the resulting hexane layers were pooled, concentrated and reconstituted with known quantity of HPLC grade hexane. 10 μl of extract was subjected to HPLC analysis and CoQ_{10} was estimated in the sample as mg l^{-1}. The specific CoQ_{10} content was expressed as mg g^{-1} of DCW by dividing mg l^{-1} activity by g l^{-1} of DCW.

Quantification of CoQ_{10}

CoQ_{10} was estimated by high performance liquid chromatography (HPLC, Agilent Make) using normal phase Kromasil Silica column (250 × 4.6 mm, 5 μm particle size). The mobile phase consisted of Hexane: Isopropyl alcohol (95:5) and used in isocratic mode with the flow rate of 1 ml min^{-1}. The detection was carried out at 275 nm. The authentic CoQ_{10} (Sigma-C9538) was used as a reference standard.

Microscopic Observation (Confocal, SEM)

The wet mounts of the cultures were observed under laser confocal microscope (LSM700-Zeiss) at 40x magnification. The 24 h grown slide cultures [28] of the wild type and mutant UF16 on YM agar were observed under FEI Quanta 200 environmental scanning electron microscope (SEM) under low vacuum mode at 5,000x magnification.

Physiological and Phenotypic Characterization

The physiological characterization in terms of utilization of different substrates and growth conditions was carried out by standard methods used in classical taxonomy as described [29]. Carbohydrate assimilation tests were carried out using Disc Method (Discs from Hi-Media, India). Agar plates containing yeast nitrogen base (HiMedia, India) were surface seeded with 50 μl cell suspension (10^8 cfu ml^{-1}) and allowed to dry completely. The carbohydrate discs (Hi-Media, India) were placed and pressed gently on the surface of the plate at sufficient distance (2 cm) from each other. Incubation was carried out at 30 ± 1.0 °C for 18–48 h and observed for the presence of growth around the discs. The atorvastatin sensitivity was tested by plating strains on YM agar plate containing atorvastatin (40 μg ml^{-1}). The auxotrophy was ruled out by plating the strains on minimal dextrose agar medium [30].

Molecular Characterization Using PCR Amplification, Sequencing and Genetic Analysis of Large Subunit (LSU) and Small Subunit (SSU) Region of rRNA Gene

The yeast genomic DNA of wild type and mutant strain UF16 was extracted as per reported protocol [31]. The PCR amplification of 25S large subunit (LSU) of rRNA gene was performed using primer 5.8SR (5′TCGATGAAGAACG CAGCG3′) and LR7 (5′TACTACCACCAAGATCT3′). The PCR was performed at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min 30 s; and 72 °C for 10 min. The PCR amplification of 18S small subunit (SSU) of rRNA gene was performed using primer 1F (5′CTGGTGCCAGCAGCCGCGGYAA3′) and 4R (5′CKRAGGGCATYACWGACCTGTTAT3′). The PCR was performed at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min 30 s; and 72 °C for 10 min. The PCR product was checked on 1 % agarose gel. The 1 kb ladder was used as a reference (Fermentas International Inc, Canada). The PCR product was purified and sequenced using ABI Big Dye Terminator V3.1 cycle sequencing reaction kit (Applied Biosystems, USA). The sequencing analysis was performed using V5.2 (Applied Biosystems, USA). Homology search was performed using the web-based BLAST service provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The sequences obtained for wild type and mutant strain were deposited in GenBank. The sequence alignment of all closest hits was performed using ClustalW [32] and phylogenetic analysis was performed using MEGA5 software [33]. The comparison of sequences was carried out to detect the presence of mutations at any position in the sequence. The evolutionary history was inferred using the UPGMA method [34]. The evolutionary distances were computed using the Maximum Composite Likelihood method [35]. The clustering of associated taxa in phylogenetic tree was performed using bootstrap test [36].

Random Amplification of Polymorphic DNA (RAPD) Analysis

The yeast genomic DNA of wild type and mutant strain UF16 was extracted as per reported protocol [31]. The six numbers of arbitrarily designed, 10-mer oligonucleotide primers used in this study and their melting temperature (Tm) values are listed in Table 1. Each RAPD reaction mixture contained 25 ng of genomic DNA; 20 pM the appropriate primer; 200 pM (each) dATP, dCTP, dGTP and dTTP and 1.0 U of Taq DNA polymerase in the PCR buffer (geneOmbio technologies, Pune). PCR conditions included initial denaturation at 94 °C for 10 min, followed by 40 cycles of 94 °C for 1 min, 39 °C for 1 min 30 s, 72 °C for 2 min. Final extension was performed at 72° C for 10 min. Reaction product was analyzed by electrophoresis using 1.5 % (wt/vol) agarose (Promega Corporation, Madison, WI 53711 USA) and gel slabs (10 cm × 16 cm × 6 mm) with 0.5x Tris–borate- EDTA buffer. Gels were stained with ethidium bromide, placed over a source of UV light, and then photographed. The molecular sizes of DNA fragments, relative to molecular size standards gScale 100 bp (geneOmbio technologies, Pune) and GeneRuler 1 kb Ladder (Fermentas International Inc, Canada), were determined. The analysis of RAPD gel was performed using *Quantity One*® 1-D analysis *software* on a GelDoc-XR System (Bio-Rad Inc. CA). Molecular size mapping of bands generated in RAPD profile was carried out using the same software and based on the data, common and polymorphic bands were scored. For comparison of band pattern and generating phylogenetic tree for wild type and mutant strain, we included isolated *Aspergillus* sp. as out-group strain and used the band pattern obtained for *Aspergillus* sp. using similar primers that have been used in the study. The binary matrix of the amplicons was designed in the form of 1 and 0 for phylogenetic analysis. The bands were scored 1 for presence, 0 for absence and entered into a data matrix [25]. UPGMA based phylogenetic tree was constructed using MEGA5 software [33].

Table 1 Melting temperature (Tm) and sequences of different primers used in RAPD analysis

Primer	Melting temperature (Tm) (°C)	Sequence
GBO-01	40.30	TCCGCTCTGG
GBO-02	41.54	CTGGGCACGA
GBO-03	43.60	TGGCGCAGTG
GBO-04	38.78	ACGCCAGAGG
GBO-05	36.96	CCTTGACGCA
GBO-06	38.00	GAGCGAGGCT

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 three trials. All error bars represent standard error of mean.

Accession Numbers of the Strains and Sequences Derived in this Study

The 25S LSU ribosomal RNA gene sequence of wild type *S. johnsonii* and mutant UF16 was derived and deposited in GenBank (Accession number JX307114.2 and JN651929.2 respectively). Similarly, 18S SSU ribosomal RNA gene

sequence of wild type and mutant UF16 was derived and deposited in GenBank (Accession number KF692019.1 and KF692020.1 respectively). The *S. johnsonii* wild type strain (ATCC 20490) and its mutant UF16 was deposited in Microbial Culture Collection (MCC) of National Center for Cell Sciences (NCCS), Pune, India under accession number MCC1092 and MCC1093 respectively.

Results and Discussion

Mutagenesis, Selection of Mutants and Screening in Shake Flask

The process of mutagenesis and rational selection has been utilized for improvement of industrially important strains for overproduction of desired metabolites [19, 20, 37]. In our previous study, an ethyl methane sulfonate (EMS) induced (exposure at 20 µl ml^{-1} for 30 min with 92 % reduction in viability), atorvastatin (20 µg ml^{-1}) tolerating mutant EA22 was generated from *S. johnsonii* ATCC-20490 with 2-fold improvement in CoQ_{10} titer (HPTLC method) [17]. EA22 showed mycelial growth forms like wild type strain but it was less pigmented and had reduced cell size. In the present study, a natural variant of this mutant (E2A22) that showed maximum 1.6-fold improvement in specific CoQ_{10} content was selected and used as a starting strain for further improvement. To overcome the limitations of HPTLC method for CoQ_{10} estimation, in terms of accuracy when used for primary screening of mutants [17], the HPLC method was followed for subsequent screenings. E2A22 was subjected to combined exposure of UV and free radicals generated by photodynamic action of methionine-riboflavin (MR) mixture [38]. In this method the antioxidant property of CoQ_{10} was exploited [3]. Carotenoid pigments and CoQ_{10} were found to be the major antioxidant component of *Sporobolomyces salmonicolor* strain [39]. The UV mutated cells were simultaneously exposed to free radicals, expecting the survivors may overcome the oxidative stress caused by free radicals by improved flux of cellular antioxidants. Mutagenesis and selection for improved production of CoQ_{10} in *Sporidiobolus ruineniae*, based on survival at free radical generating growth inhibiting conditions has been reported. Mutants were selected on hydrogen peroxide and paraquat that generates free radicals mediated oxidative stress [40].

Photodynamic action of methionine-riboflavin mixture (MR) with a high level of reactive oxygen species (ROS) production is a powerful microbicidal system. Under illumination, riboflavin could be excited by light to a triplet state and thereafter activate oxygen and methionine in the mixture by energy transfer [27]. This activation process thus produces an abundant amount of toxic materials and incurs a strong biocidal activity. The oxidative damages on cellular lipids, proteins and DNA are central factors causing cell death by ROS [38]. It is reported that MR photodynamic action kills many plant pathogens and produces high levels of ROS [41]. Also, the naturally occurring, intracellular free radical mutations especially in mitochondria, has been reported [42].

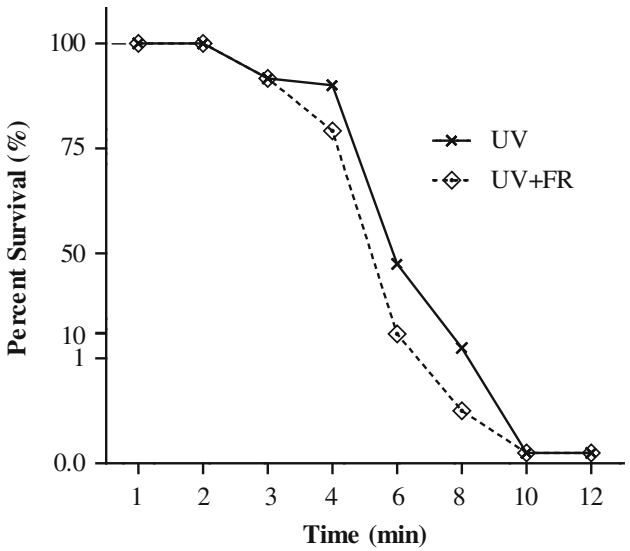

Fig. 1 Reduction in percent survival with time by combined action of UV and free radicals generated by photodynamic action of methionine-riboflavin mixture (MR) on mutant E2A22. At 6 min exposure, combined action resulted in significant reduction in cell viability as compared to UV alone (values are average of three trials)

The formation of free radicals soon after exposure to MR mixture was confirmed using nitro blue tetrazolium dye which turns blue in presence of free radicals [38]. From the pilot trials, fluorescent tube source took more than 3 min to generate ROS from MR mixture whereas UV rays (254 nm) showed instant action. Since UV source generates faster photodynamic action of MR mixture than fluorescent tubes, attempts were made to create mutations in E2A22 as well as to allow selection of mutants based on tolerance to free radicals using combined action of UV as well as free radicals. The exposure conditions for mutagenesis by UV rays and subsequent lethal action of ROS, were optimized based on the reduction in cell viability above 90 %. Figure 1 shows the percentage reduction in cell viability of mutant E2A22 upon exposure to UV as well as combined action of UV and free radicals at different time points. Around 90 % reduction of cell viability was observed at 6 min exposure during combined action, whereas UV alone showed significantly less reduction in viability (55 %) at that time indicating lethal action of free radicals on the cells. Beyond 8 min, the UV alone showed maximum reduction (>95 %) in viability hence mutants were

selected from 6 min exposed suspension by plating on atorvastatin (20 and 40 μg ml^{-1}) containing plates. Most of the mutant colonies on atorvastatin containing plate were mucoid and pale in color. The toxicity of atorvastatin to yeast cells has been reported [43] hence mutant colonies on the atorvastatin containing plate were picked up and further maintained on plain YM agar plates without atorvastatin.

CoQ_{10} is an isoprenylated benzoquinone and its biosynthesis involves three major steps consisting of synthesis of quinone ring (*para*-hydroxy benzoic acid), synthesis of isoprene chain (decaprenyl diphosphate) followed by quinonoid ring modification [4]. In yeasts, the isoprene units of CoQ_{10} are derived from mevalonate (isoprenoid) pathway [44]. Statins are competitive inhibitors of HMG-CoA reductase, which catalyses the conversion of HMG-CoA to mevalonate, a rate-limiting step in isoprenoid biosynthetic pathway involved in synthesis of CoQ_{10} [18, 45]. Enhanced production of CoQ_{10} in *Schizosaccharomyces pombe* have been reported by overexpressing HMG-CoA reductase gene and induction with arachidonic acid [46]. Hence the approach of selecting mutant strains on atorvastatin was followed as it was hypothesized that induced mutations leading to atorvastatin resistance in *S. johnsonii* may induce upregulation of mevalonate pathway by improving flux of isoprene units for improved CoQ_{10} production.

Through primary screening of more than 240 mutants, the best ones were selected and subjected to reproducibility testing in the flask for three consecutive batches. In the course of study, mutant UF16 which was selected on atorvastatin (40 μg ml^{-1}) was found to be the best mutant strain. The performance of two mutants namely, mutant E2A22 and UF16 was compared with wild type strain in YMP medium and results are expressed in Fig. 2. Mutant UF16 produced almost 10 % less DCW than wild type and showed significant improvement in CoQ_{10} titer and specific content (Supplementary Fig. 1). Mutant E2A22 did not show reduction in DCW. The improved mutant strain UF16, generated in the study produced 7.4 ± 0.88 mg l^{-1} of CoQ_{10} on YMP medium at 96 h, having corresponding specific CoQ_{10} content of 0.749 ± 0.021 mg g^{-1} of DCW which was 2.3-fold higher than the wild type strain. Hence the mutation and selection based on combined action of UV and free radicals was found to be useful in generating a mutant with significant improvement in CoQ_{10} content. The CoQ_{10} yield in UF16 can be further improved by fermentation media modification and process optimization. This strain was found to be stable through the continuous propagations for more than 10 passages. Although, specific CoQ_{10} content of UF16 has been improved, it is still less as compared to the other high CoQ_{10} producing bacterial strains, with maximum reported value of 11.84 mg g^{-1} of DCW [2]. Hence efforts are needed to further improve this strain by mutagenesis or metabolic engineering techniques.

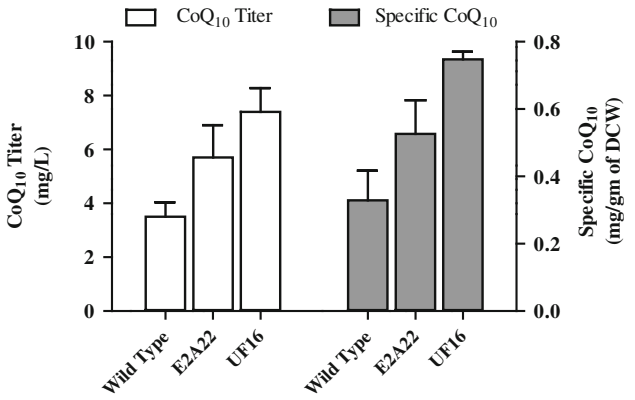

Fig. 2 Comparison of CoQ_{10} titer (mg l^{-1}) and specific CoQ_{10} content (mg g^{-1} of DCW) between wild type strain and mutant strains. Mutant UF16 showing significant improvement in CoQ_{10} content

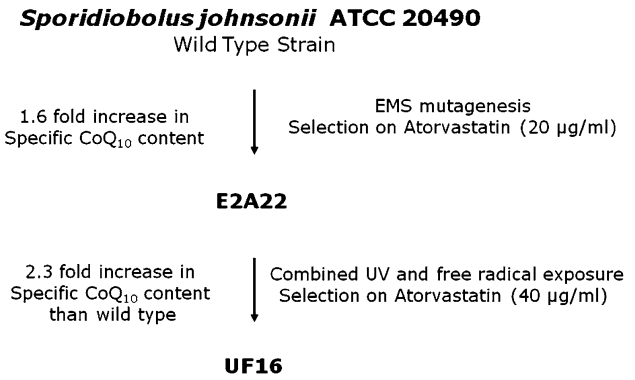

Fig. 3 Genealogy of mutant UF16 derived from *Sporidiobolus johnsonii* ATCC 20490 by sequential mutagenesis and selection on atorvastatin containing plate

Morphological and Physiological Characterization of Mutant UF16

The genealogy of CoQ_{10} overproducing mutant strain UF16 was shown in Fig. 3. The mutant UF16 showed difference in colony morphology where the colonies were bigger, mucoid and less pigmented than wild type strain as shown in Fig. 4a, b. Due to reduced pigmentation, UF16 was thought to be deficient in carotenoid production. The reduced accumulation of carotenoid pigments in enhanced CoQ_{10} producing *R. sphaeroides* [14, 47] indicated the allocation of more cellular isoprenoid resources towards CoQ_{10} production. Further investigation is required to correlate the relative distribution of isoprenoid flux to carotenoids and CoQ_{10} biosynthetic pathway in UF16. This

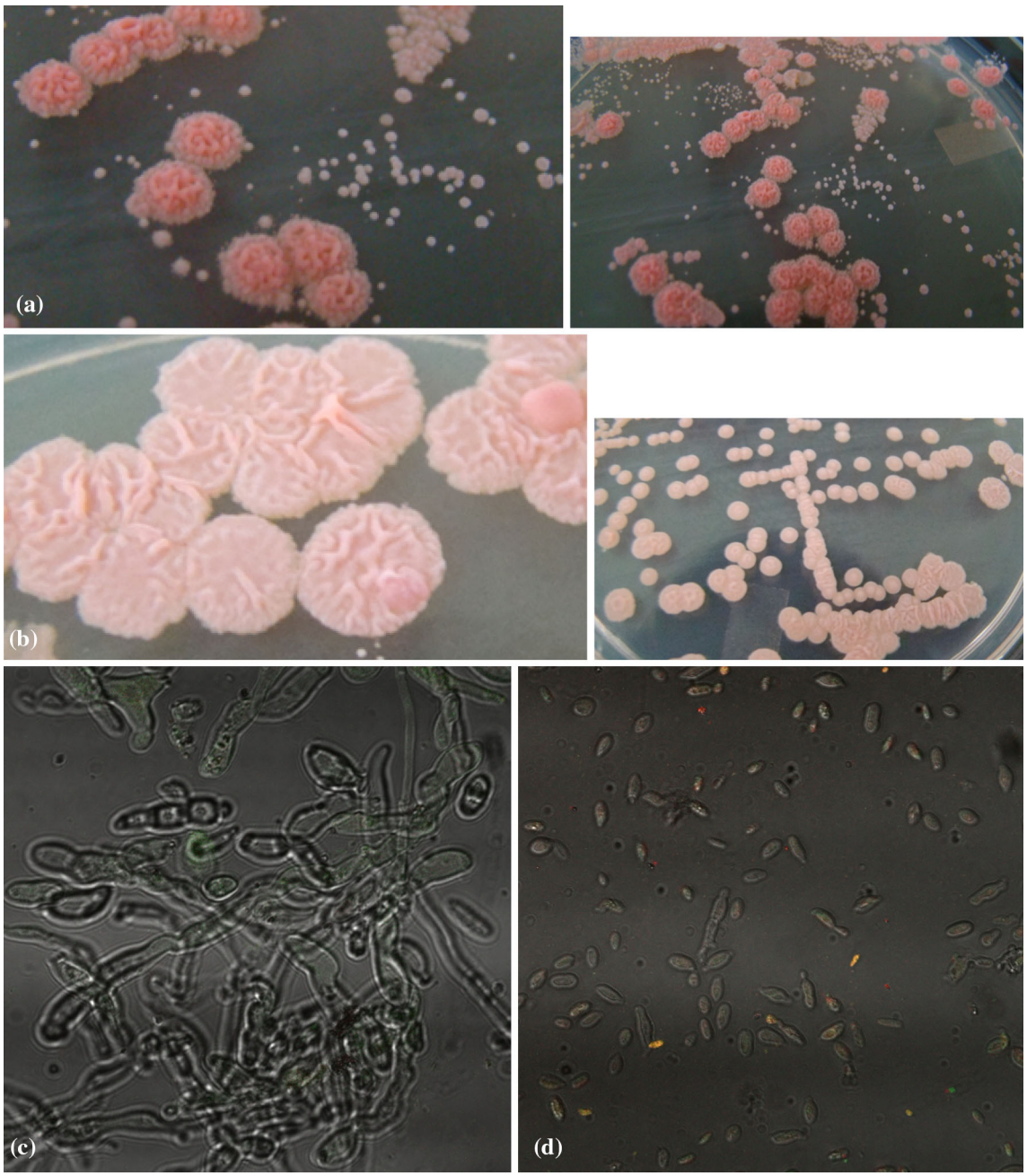

Fig. 4 Morphological (cultural and microscopic) differences between wild type and mutant UF16 (2 week old cultures on YM agar plate) **a** Red pigmented wild type colonies with few young colonies that appeared late after germination of discharged ballistospores. **b** Big, mucoid and less pigmented colonies of UF16. **c** Wild type strain showing mycelial growth and few big size yeast forms (40x magnification). **d** UF16 strain showing small size yeast cells and scanty mycelial forms (40x magnification)

observation may be useful in future strain improvement process on this strain, so that less pigmented mutants or mutants blocked in pigment biosynthesis, would be preferred as a means of rational selection for high CoQ_{10} producers.

Microscopically, from 2 week old cultures on YM agar plate, wild type strain showed mycelial growth whereas UF16 showed mainly yeast forms with reduced size and scanty mycelial forms (Fig. 4c, d). The reduction in the size of UF16 cells was confirmed by scanning electron microscopic observation (Fig. 5a, b) and by confocal microscope (Fig. 5c, d). For this purpose, very young cells in yeast form were observed before the formation of mycelia in wild type. The wild type cells were elongated whereas UF16 showed oval shaped yeast cells. The average size of wild type cells (8.44 μm length, 3.05 μm width) and

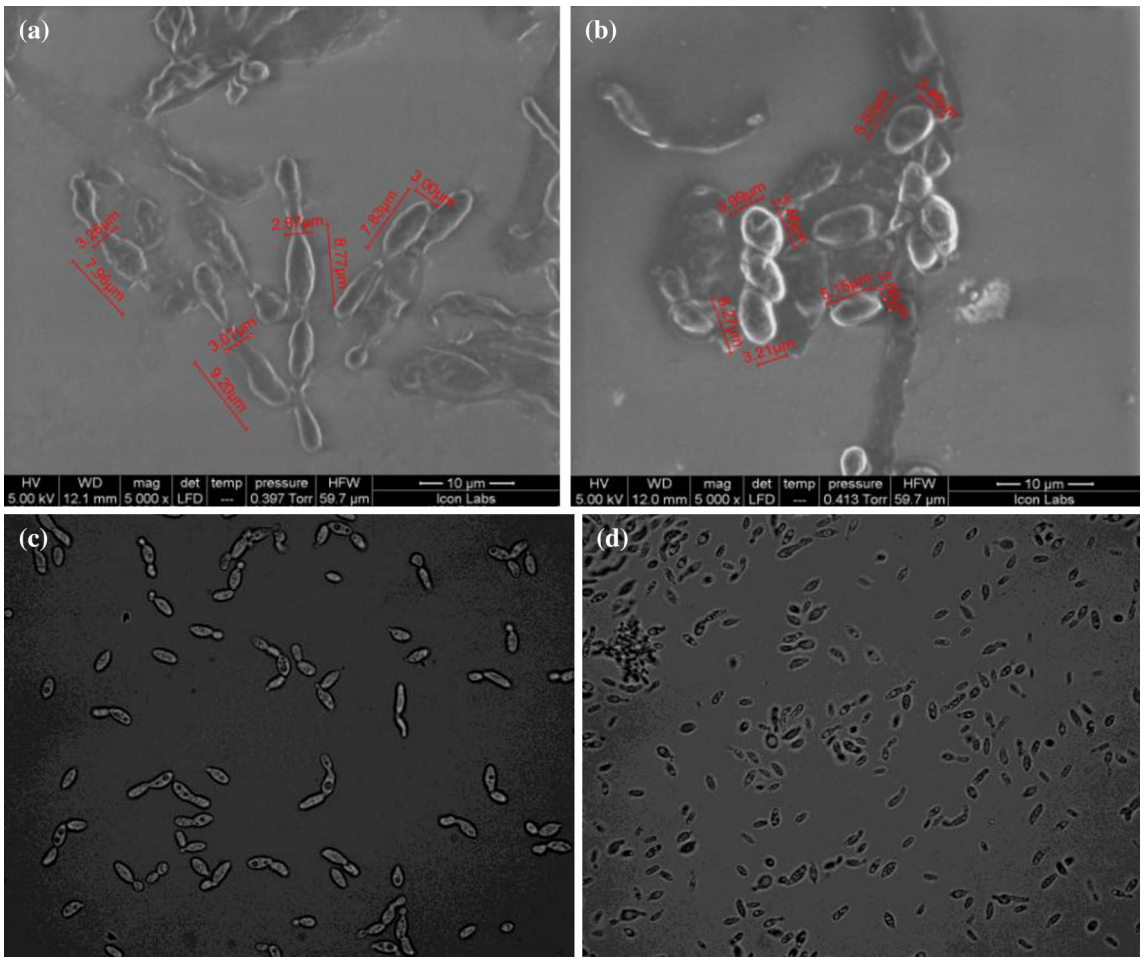

Fig. 5 Scanning electron microscopic images of wild type (**a**) and UF16 (**b**) cells grown for 24 h on slide culture, showing difference in size and shape (5000x magnification). Confocal microscopic images of wild type (**c**) and UF16 (**d**) cells grown for 24 h on YM agar plate, showing difference in size and shape (40x magnification)

that of UF16 (5.6 µm length, 3.33 µm width) was estimated using SEM, which revealed that UF16 has reduced size. Under submerged cultivation on seed and production medium, wild type strain showed mycelial forms and UF16 showed mainly yeast cells with significant reduction in mycelial growth forms (Fig. 6). UF16 showed partial inhibition of mycelia formation in both surface and submerged cultivation. On YM agar plate, wild type strain showed formation of small colonies around the main colonies (Fig. 4a) after 8–10 days of incubation, which are formed by germination of violently discharged ballistospores [30], and such colonies were not observed in UF16 even after 4–8 weeks incubation. Life cycle of *S. johnsonii* has been reported and it was found to contain dikaryotic mycelia stage arising from diploid yeast cells [30, 48]. Unlike wild type strain, UF16 did not show abundance of mycelial forms. Investigation of the life cycle of UF16 would throw a light on its morphological changes.

Physiological characterization based on growth characteristics and assimilation of substrates was carried out and results are expressed in Tables 2, 3. UF16 showed similar profile of substrate utilization and growth pattern as compared to wild type strain. Tallman and Fell [49] described the physiological characteristics of *S. johnsonii* and differentiated it from other *Sporidiobolus* species based on melezitose and nitrate assimilation. The UF16 and wild type strain showed positive test for both, melezitose and nitrate assimilation as reported in literature. Overall, physiological tests did not reveal any differences between wild type and UF16. UF16 was able to grow on minimal dextrose agar medium, hence auxotrophy was ruled out. UF16 showed luxuriant growth on YM agar plate containing 40 µg ml^{-1} of atorvastatin whereas wild type strain showed no signs of growth. This observation confirmed the resistance pattern of UF16 to atorvastatin. Phenotypically, UF16 was very well differentiated from wild type strain.

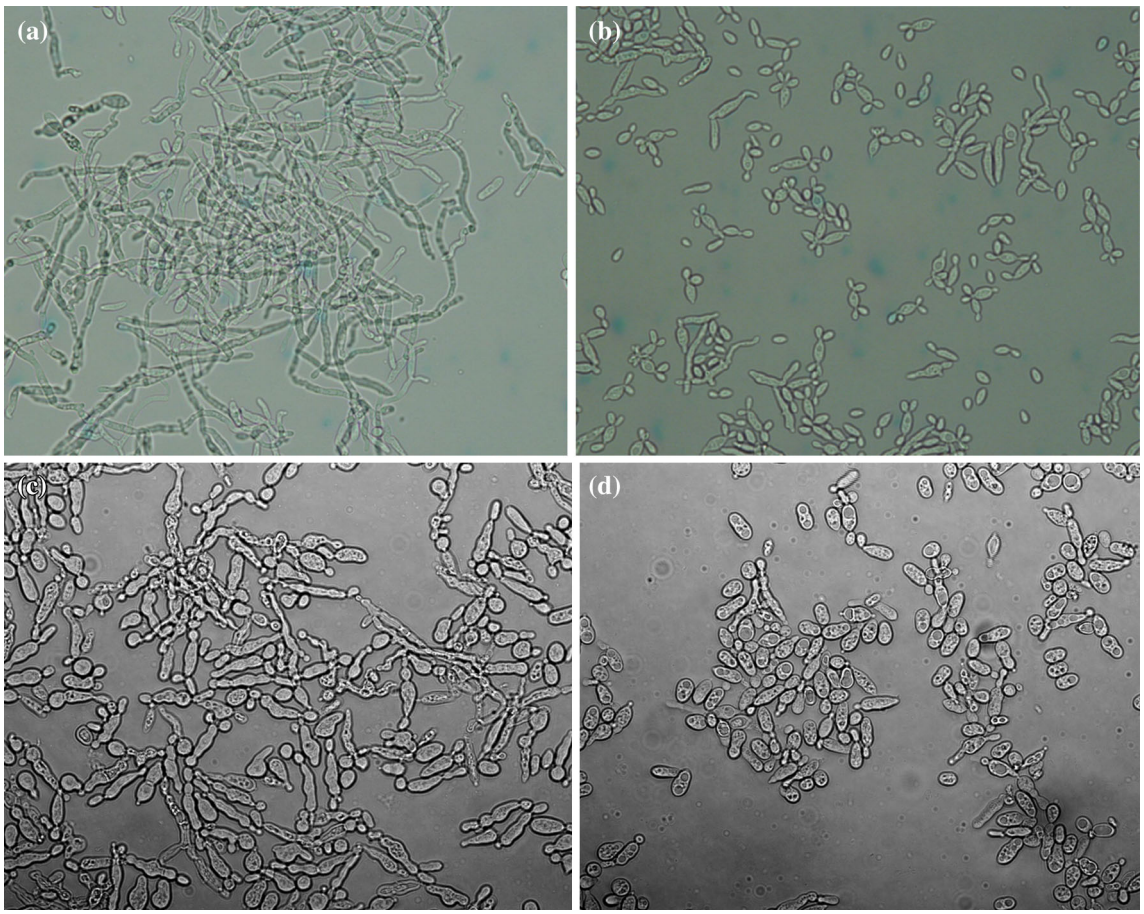

Fig. 6 Microscopic observation of Wild type and UF16 growth under submerged cultivation: **a** Wild type strain in seed medium (24 h) showing complete mycelial growth, **b** UF16 in seed medium (24 h) showing mostly budding cells and few mycelia. **c** Wild type in production medium (96 h) showing mycelial forms **d** UF16 in production medium (96 h) showing mostly yeast cells (40x magnification)

Molecular Characterization of Mutant UF16 Based on rRNA Gene Sequences

In addition to phenotypic differentiation, the characterization of mutant strain at molecular level is essential to detect genotypic changes at nucleotide base pair level. The whole genome sequences of *S. johnsonii* have not been reported so far and only few sequences of rRNA gene are available in the databases which are used for molecular identification and phylogenetic inference [50, 51]. In the present study, mutant UF16 was characterized based on rRNA gene sequences and investigations of mutations on these sequences were carried out.

The phylogenetic tree constructed using SSU and LSU rRNA gene sequences of wild type, UF16 and closely related strains is shown in Figs. 7, 8. It was observed that in case of tree constructed using 25S LSU rRNA gene sequences (Fig. 7); UF16 formed a distinct subclade in the tree together with wild type strain, supported by 100 % bootstrap value in UPGMA analysis, thus separating them from each other. The phylogenetic tree based on 18S rRNA gene sequences (Fig. 8) also exhibited differences between two strains. In the BLAST search (NCBI), the LSU rRNA gene sequences of wild type strain showed 100 % similarity with isolated strain of *S. johnsonii* and not with UF16. The multiple sequence alignment of large subunit rRNA sequence of wild type (JX307114.2) and UF16 (JN651929.2) was carried out using ClustalW (Supplementary Fig. 2). UF16 was found to have mutations on seven nucleotide base pairs which are either transition, transversion or deletion mutation. Few mutations were also observed on 18S small subunit rRNA sequence of UF16 after sequence alignment (Supplementary Fig. 3). Usually rRNA sequences are conserved and used for identification of strains till species level. However, the mutations detected on rRNA gene of UF16 were less than 1 % of total base pairs and its sequence showed 99 % homology with the wild type strain. The transition, transversion and deletion type of mutations detected on UF16 genes indicate the cause of mutagens used to generate UF16. These mutations

Table 2 Physiological characterization based on growth characteristics and assimilation tests

Test	Wild type	Mutant UF16
Raffinose	–	–
Urease	+	+
Gelatin	–	–
Citrate	+	+
Methanol	–	–
Ethanol	+	+
Glycerol	+	+
Nitrate	+	+
Soluble Starch	–	–
Starch Formation	–	–
Melezitose	+	+
Erythritol	–	–
N-acetyl-D-glucosamine	–	–
D-Gluconate	+	+
α-Methyl-D-glucoside	+	+
10 % NaCl/5 % glucose	+	+

(+) Positive, (−) negative

Table 3 Carbohydrate assimilation test using disc method (Hi-Media, India)

Carbohydrate	Wild type	Mutant UF16
Inositol	–	–
Mannitol	+	+
Cellobiose	+	+
Xylose	+	+
Sorbitol	+	+
Melibiose	–	–
Fructose	+	+
Maltose	+	+
Dextrose	+	+
Adonitol	+	+
Sucrose	+	+
Arabinose	+	+
Rhamnose	–	–
Dulcitol	–	–
Lactose	–	–
Galactose	–	–
Inulin	–	–
Salicin	+	+
Mannose	+	+
Trehalose	+	+

(+) Positive, (−) negative

in UF16 could not be linked to phenotypic or genotypic changes on CoQ_{10} biosynthetic pathway that resulted in improved yield. However, investigation of mutations responsible for deregulation of pathways leading to improved CoQ_{10} production in UF16 is essential and could be achieved using full genome sequencing as well as gene expression studies, especially from CoQ_{10} pathway genes. The mutations in UF16 displayed the novelty of the strain resulted from the mutagenesis process.

Molecular Characterization of Mutant UF16 Based on RAPD Analysis

For rapid differentiation of mutant strain, DNA fingerprinting method by RAPD was established. RAPD is proved to be a rapid molecular method for generating whole genomic DNA fingerprints which are species or even strain specific [29, 51, 52]. In this study, the RAPD analysis was conducted for differentiating mutant UF16 from wild type strain, taking account of its whole genome. Initially we screened twenty oligonucleotide primers for amplification of yeast DNA. The RAPD analysis was performed on wild type and mutant UF16 using six selected primers (Table 1) that could successfully amplify the DNA. The RAPD band profile obtained is shown in Fig. 9. For each primer, the array of DNA fragments consisted of bands of high or low intensities of ethidium bromide staining, were identified using the band density analysis tool of the software (*Quantity One*®, Bio-Rad Inc. CA.). The size estimation of each fragment was also performed using the same software through molecular weight estimation tool (Supplementary Fig. 4) thereby accurately sorting the polymorphic bands (Supplementary Fig. 5). With each primer, the profile of DNA fragments obtained with wild type and UF16 showed DNA fragment length polymorphism along with several similar sized fragments. The reproducibility of the RAPD profile was checked with replicated DNA preparation and also by using purified ultra diluted (1:100) RAPD products as template for replicate experiments, which showed identical pattern of DNA fragments.

By and large, DNA fingerprinting using six RAPD primers showed 34 % of the amplified polymorphic fragments indicating the presence of intraspecific polymorphism between the two strains. The amplicon profile obtained with two strains using six different primers and its genetic analysis is shown in Table 4. To estimate the values of similarity coefficient (SC), the formula proposed by Nei and Li [53] was used to generate computer algorithm and transformed into genetic distance (GD) analysis profile [54]. Out of six primers used, GBO-2 and GBO-5 showed significantly lower values for SC indicating greater GD between the parent and mutant strain. Genetic analysis suggests that the wild type and mutant strain exhibit genetic diversity with respect to RAPD pattern obtained with the six arbitrarily designed 10-mer oligonucleotide primers used in the study. The maximum GD was observed

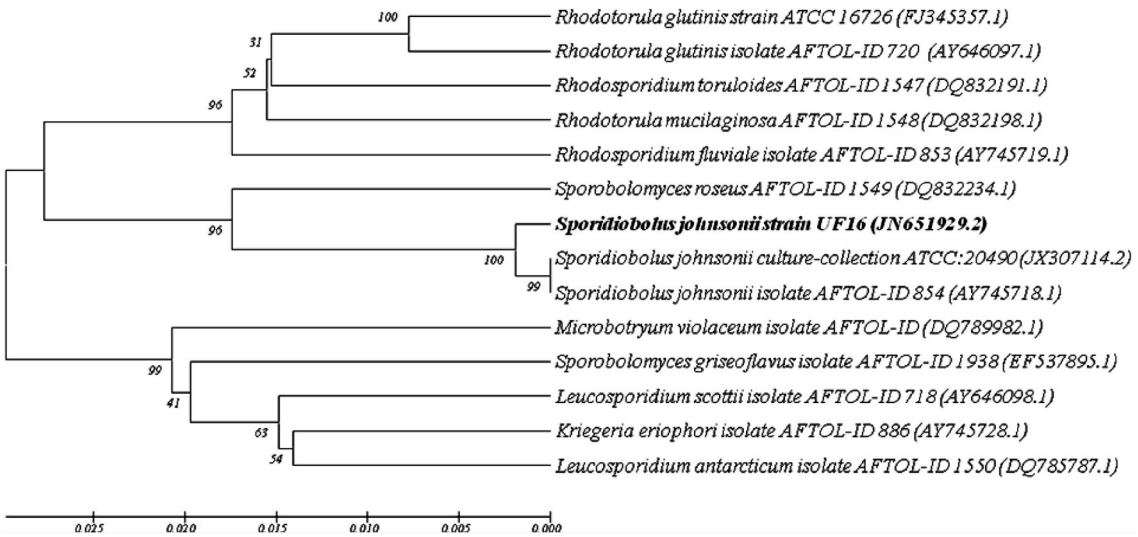

Fig. 7 Phylogenetic tree based on large subunit rRNA gene sequences, showing relationship between *S. johnsonii* ATCC-20490, mutant UF16 and closely related gene sequences. Bootstrap values expressed as percentage of 500 replications are given at nodes. The evolutionary history was inferred using the UPGMA method [34]. The optimal tree with the sum of branch length = 0.23174996 is shown. [36]. The evolutionary distances were computed using the Maximum Composite Likelihood method [35] and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 [33]

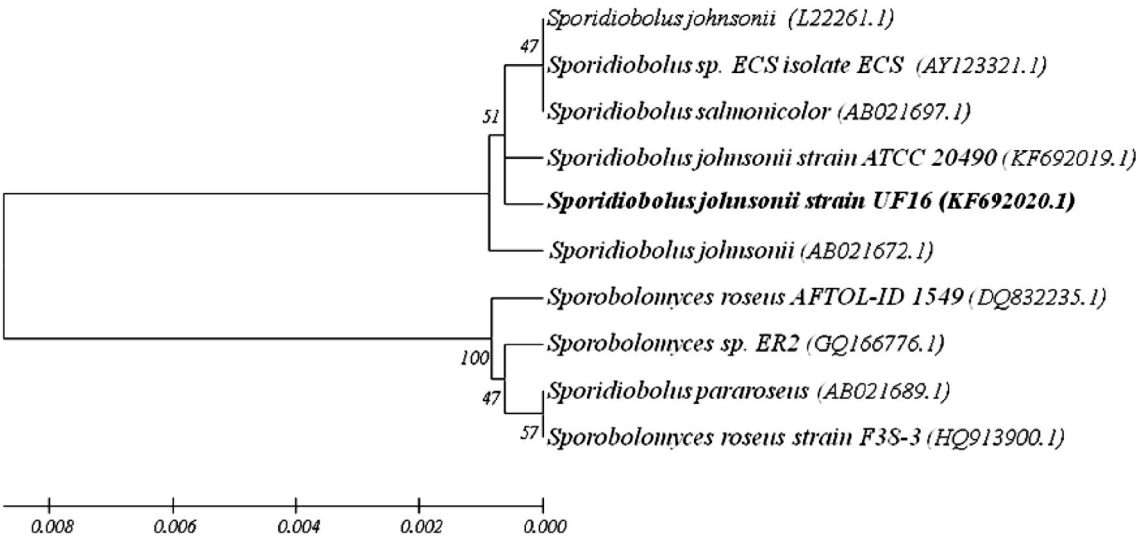

Fig. 8 Phylogenetic tree based on 18S rRNA gene sequences, showing relationship between *S. johnsonii* ATCC-20490, mutant UF16 and closely related gene sequences. Bootstrap values expressed as percentage of 500 replications are given at nodes. The evolutionary history was inferred using the UPGMA method [34]. The optimal tree with the sum of branch length = 0.02100761 is shown [36]. The evolutionary distances were computed using the Maximum Composite Likelihood method [35] and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 [33]

with primer GBO-2 which was 0.56 whereas the mean GD computed through analysis of data obtained with all six primers was found to be 0.235. The phylogenetic tree constructed from the RAPD band pattern obtained from wild type, UF16 and out-group strain *Aspergillus* sp. (Supplementary Fig. 6) is shown in Fig. 10. The tree confirms the significant genetic diversity between wild type and UF16 as both of them have been placed on different subclade at significant distance. The PCR-fingerprinting method of RAPD could detect the genetic variation in UF16, may be due to large number of point mutations on genome, out of which few have been detected on rRNA gene sequences.

Although RAPD analysis was found to be unreliable at least as a means of identifying yeasts in ecological studies [55], it is still effectively used for discriminating the

strains genetically. This method has been used to detect genetic diversity among the shuffled strains of *Streptomyces avermitilis* NEAU1069 showing improved doramectin production [56]. Schlick et al. [57] used RAPD markers to differentiate the gamma rays induced mutant strains of *Trichoderma harzianum*. The diesel-

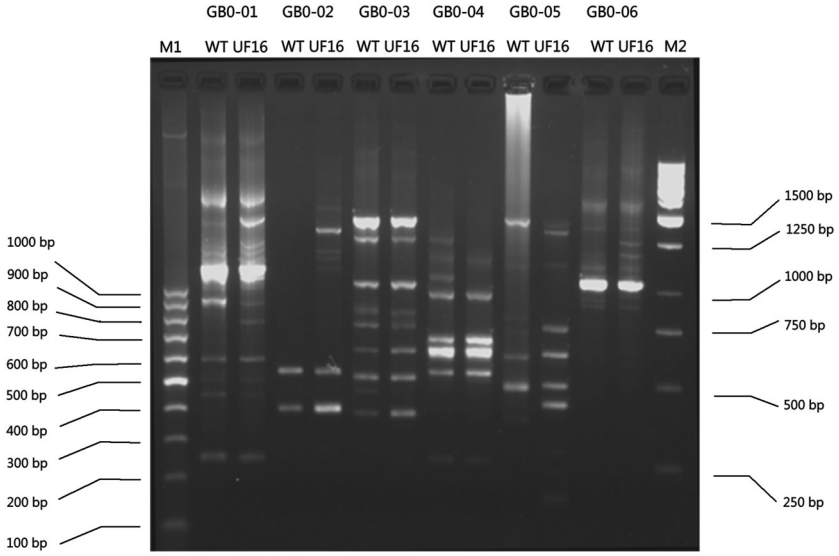

Fig. 9 RAPD profile of wild type (WT) and mutant (UF16) strain obtained from six different primers (GBO-01 to GBO-06): Lane M1- 100 bp DNA Marker; Lane WT—RAPD amplicon from wild type; Lane UF16—RAPD amplicon from Mutant strain UF16; Lane M2—1 kb DNA marker

Table 4 RAPD amplicon profile obtained with wild type and mutant UF16 strain with genetic analysis

Name of the Primer	No. of amplicon in wild type strain (Nx)	No. of amplicon in mutant UF16 (Ny)	No. of common amplicon (Nxy)	No. of polymorphic bands	Similarity Coefficient* SC = 2Nxy/(Nx + Ny)	Genetic distance GD = 1 − SC
GBO-01	7	9	7	2	0.88	0.13
GBO-02	2	7	2	5	0.44	0.56
GBO-03	9	8	8	1	0.94	0.06
GBO-04	8	6	6	2	0.86	0.14
GBO-05	5	8	4	5	0.62	0.38
GBO-06	6	8	6	2	0.86	0.14

* Nei and Li [53]

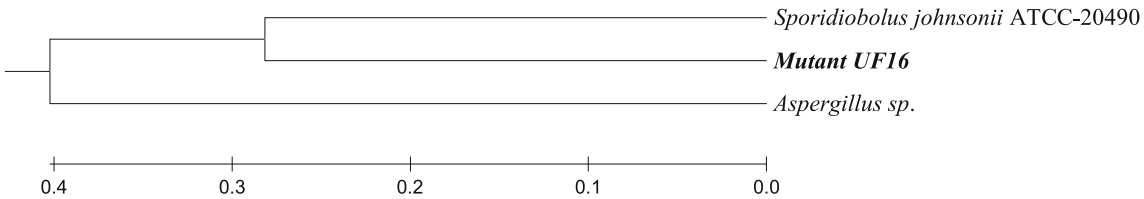

Fig. 10 Evolutionary relationships of taxa based on RAPD profile: The evolutionary history was inferred using the UPGMA method [34]. The optimal tree with the sum of branch length = 1.08627650 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [35] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 47 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [33]

degrading and carotenoid producing novel bacterial strain was distinguished successfully from the strain showing closest similarity with its 16S rRNA gene sequence, based on differences in RAPD fingerprinting pattern [58]. The genetic differences between the wild type and the mutants of *Metarhizium anisopliae* have been detected by RAPD [59]. The RAPD technique was also used on malarial parasite *Plasmodium falciparum* to detect genetic markers for chloroquine-resistant strains [60]. Other than differentiating the strains, RAPD profile has also been used as a tool to confirm genetic similarity between two fungal strains [61].

Conclusion

During sequential mutagenesis process, the combined action of UV rays and free radicals resulted in generation of significantly higher CoQ_{10} producing *S. johnsonii* mutant UF16 that also showed tolerance to higher concentration of atorvastatin. UF16 produced 7.4 ± 0.88 mg l^{-1} of CoQ_{10} on YMP medium at 96 h, having corresponding specific CoQ_{10} content of 0.749 ± 0.021 mg g^{-1} of DCW which was 2.3-fold higher than the wild type strain. UF16 showed prominent alteration in cellular morphology. In UF16, the mutations were detected on nucleotide base pairs of rRNA gene sequences. Detecting the mutations on whole genome and correlating its beneficial effect on CoQ_{10} biosynthesis is essential. RAPD analysis was found useful in rapidly discriminating the UF16 from wild type strain at molecular level. Hence this method may be useful in future strain improvement process on this strain to rapidly differentiate the generated high yielding strains. It may also be possible to select the promising mutants for genome sequencing and gene expression studies, based on maximum GD in RAPD analysis as well as high CoQ_{10} content. The common and polymorphic bands generated in RAPD analysis may be useful to develop strain specific sequenced characterized amplified region markers. Overall it can be concluded that the CoQ_{10} overproducing mutant UF16 is morphologically and genetically different from wild type strain, hence characterized as a novel strain of *S. johnsonii*. Investigating the changes on CoQ_{10} biosynthetic pathway genes and their related gene expression may be desirable to understand the cause of CoQ_{10} overproduction in UF16.

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