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Protoplast fusion technology for improved production of coenzyme Q10 using *Paracoccus denitrificans* ATCC 19367 mutant strains

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Abstract

Induced mutants generated from *Paracoccus denitrificans* ATCC 19367 having antibiotic resistant markers, were used as parent strains to carry out protoplast fusion. The generated fusants were screened using standardized protocol for CoQ_{10} production. Among the generated fusants, one fusant namely PF-P1 showed 1.73 folds enhancements in specific CoQ_{10} content than wild type strain. Fusant PF-P1 was characterized by biochemical and molecular approaches where it showed differences than wild type strain. The fusant was further identified by 16S rRNA gene sequence analysis that showed eight nucleotide base pair mutation on conserved region and 99% homology with *Paracoccus denitrificans* strains.

Keywords: *Paracoccus denitrificans*, protoplast fusion, AFLP, 16S rRNA sequence, phylogenetic analysis.

Introduction

Coenzyme Q_{10} (CoQ_{10}) is a benzoquinone containing a 10-unit isoprene side chain which contributes in the oxidative phosphorylation (Wolf et al. 1958). It is widely distributed in mitochondrial inner membrane, lysosomes, peroxisomes and microsomes throughout the eukaryotic cells and is located in plasma membrane of the prokaryotic cells (Ernster and Dallner, 1995). Its main function is to transfer electrons from complex I/II to cytochrome *bc1* complex during oxidative phosphorylation for ATP generation (Kawamakai, 2002). In addition to its central role in the mitochondrial respiratory chain (Soballe and Pool, 1999), CoQ_{10} is involved in aspects of cellular metabolism and also acts as a potent antioxidant. Recently CoQ_{10} is used as an oral nutritional supplement and is beneficial in the treatment of several human diseases such as neurodegenerative, mitochondrial, cancer and heart diseases (Overvad et al. 1999; Singh et al. 1998**)**.

Due to the increased demand for CoQ_{10} as an active ingredient in formulations, several processes have been developed for its commercial production. One such is the chemical synthesis utilizing solanesol, which is a multistep reaction and not cost effective (Keinan and Eren, 1998). The economical production of CoQ_{10} using biological processes is becoming widespread. The biological process predominantly depends on microbes that produce high levels of CoQ_{10} naturally. CoQ_{10} containing bacteria include *Agrobacterium tumefaciens, Paracoccus dentrificans, Cryptococcus laurentii, Tricosporon sp., Sporobolomyces salmonicolor* and *Rhodobacter sphaeroides* (Kondo et al. 1973). These have been used to optimize the fermentation process for production of CoQ_{10}.

In case of CoQ_{10} production using microorganisms, the titers obtained with wild type strains are insufficient and there are very few reports on the mutation and strain development with respect to CoQ_{10} production (Ranadive et al. 2011). The production of CoQ_{10} using bacteria was reported and out of three bacterial strains namely *Agrobacterium tumefaciens, Paracoccus dentrificans* and *Rhodobacter sphaeroides*, *Paracoccus dentrificans* was found to produce least amount of CoQ_{10}. The chemical mutant strains derived from *Agrobacterium tumefaciens* and *Rhodobacter sphaeroides* showed improved production of CoQ_{10} (Yoshida et al. 1998). However *Paracoccus dentrificans* strain has not been explored for mutational studies leading to improvement of CoQ_{10}.

Various natural methods of recombination occur in different microorganisms, including conjugation and transduction in bacteria and heterokaryosis and the sexual cycle in fungi. Although all of these have some application in the industrial context, the most widely used methods of gene transfer for strain improvement are those which are unlikely to occur in nature-para sexual breeding (in fungi), protoplast fusion and

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genetic engineering. Recombination is a rational approach to industrial strain improvement which despite the power of the mutation and selection approach, provides a worthwhile alternative strategy. However, with the advent of protoplast fusion the situation has changed dramatically and rapid breeding techniques based on protoplast fusion are now available for a variety of industrial organisms (Rowlands, 1984; Gong et al. 2009). The technology of protoplast fusion has been used for improvement of industrial strains producing variety of metabolites. However the benefit of protoplast fusion between mutant strains of CoQ_{10} producing bacteria has not been evaluated till date.

We have selected *Paracoccus dentrificans* ATCC 19367, a wild type strain for our studies since it is known to produce CoQ_{10} and very few reports are available on genetic manipulation of this strain. The current work deals with the generation of induced mutants from *Paracoccus denitrificans* ATCC 19367 having antibiotic resistant markers followed by fusion of protoplasts prepared from mutant strains. The protoplast fusion in *Paracoccus denitrificans* strains has not been reported till date. We demonstrate the method of protoplast formation, fusion and subsequent regeneration to get the fusant strains. Attempts were made to select CoQ_{10} over producing fusant strain followed by its characterization.

Materials and methods

Producing organism

Paracoccus denitrificans ATCC 19367 was used as a starting strain to generate induced mutants and fusants. The producing strain and its mutants were maintained in the form of working stock on the Tryptic Soy Agar (TSA) slants.

Gamma (γ) Mutagenesis

Grown culture was scrapped with 0.85% NaCl to make the cell suspension of 4.8×10^8 viable cells/ml. Ten ml of cell suspension was transferred to sterile falcon tubes and exposed to the irradiation at different time period in order to get irradiation dose of 25 γ, 50 γ, 100 γ, 250 γ and 400 γ. After exposure the cell suspensions were serially diluted in sterile saline (0.85% NaCl) till 10^{-9} dilutions and 0.1 ml of suspensions were spread onto the surface of Nutrient Agar (NA) plates which were incubated at 30˚C for about 96 h. The mutants obtained from the exposure having higher kill rate, were picked up and transferred to NA plates for further use (Iftikhar et al. 2010).

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) Mutagenesis

The culture was grown overnight in 50 ml of Tryptic Soy Broth (TSB) medium in 500 ml flask to get viable count of around 10^9-10^{10} cfu/ml. Twenty ml of cell suspension was taken in sterile flask and to this 500 µl of NTG stock solution (16 mg/ml) was added to get final concentration 400 µg/ml. The flask was kept with intermittent shaking and 5 ml of sample was taken out after 10 min and 20 min exposure followed by addition of 5 ml of 5% sodium thiosulphate to arrest the activity of NTG. It was centrifuged to get pellet, reconstituted in 5 ml of 10 mM phosphate buffer (pH 7.0) and different dilutions were plated on NA plate to determine the viable count. The reduction in viable count was determined as compared to untreated suspension (Suneetha et al. 2013; Fang et al. 2009).

Selection of mutants resistant to antibiotics

Initially antibiotic sensitivity test was performed on wild type strain using different antibiotic discs (Octadisc, Hi-Media) by agar diffusion method. Neomycin, Vancomycin and Gentamycin to which the strain was most sensitive were used for selection of resistant mutants by using the gradient plate with the following concentration Neomycin (0-4 µg/ml), Vancomycin (0-2.5 µg/ml) and Gentamycin (0-3 µg/ml). The cell suspension obtained by induced mutagenesis was swabbed on to gradient plates and incubated at 30ºC for 96 h. The mutant colonies appearing on the plate above MIC gradient were picked up and transferred onto NA slants (Rowlands, 1984).

Protoplast fusion

A loopful of culture was inoculated into 500 ml flask containing 50 ml of Tryptic Soy Broth (TSB) medium and incubated on rotary shaker at 220 rpm at 30°C. Protoplasts were made from cells taken from mid of exponential phase. Five ml of broth was centrifuged at 4000 rpm for 20 min at 4°C. The supernatant was discarded and the cells were washed with 5 ml of washing buffer (composition as per given in Table 1). After first wash, the cells were sedimented and treated with 5 ml of washing buffer for different time intervals as shown in Table 1. The suspension was centrifuged to get the pellet and resuspended in buffer. Different concentrations of lysozyme was added and incubated for different time intervals as shown in Table 1. The protoplast formation was confirmed microscopically and two different protoplast suspensions from different phenotypes were mixed in 1:1 ratio (Weiss, 1976). The mixture was centrifuged at 2000 rpm for 10 min. To the pellet, 1 ml of mixture containing different concentrations of PEG, $CaCl_2$ and DMSO was added and incubated for 10-15 min. The suspension was diluted with buffer (1:1 ratio) and the suspension was serially diluted further in buffer as well as in sterile distilled water. The different dilutions were plated (0.1 ml) on regeneration medium agar plates and plates were incubated at 30°C for up to 17 days (Zhang et al. 2002; Xu et al. 2006). Different trials were taken to standardize the protocol for protoplast generation as shown in Table 1.

Screening of strains

The induced mutants and fusants were screened by shake flasks cultivation. A loopful of culture from the culture slant was aseptically transferred to the 500 ml conical flask containing 50 ml sterile seed medium consisting of 60 g of sucrose, 15 g of yeast extract, 15 g of peptone, 5 g of NaCl in 1 L demineralized water and pH was adjusted to 7.2. Flasks were kept at 30°C on a rotary shaker rotating at 220 rpm for 48 h. After 48 h, 10% (^{v}/v) of seed culture was aseptically transferred to the 500 ml conical flask containing 50 ml of sterile production medium consisting of 80 g of cane molasses, 13 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.25 g of $MgSO_4$.$7H_2O$, 40 g of corn steep liquor, 20 g of $CaCO_3$ in 1 L demineralized water and pH was adjusted to 7.2 (Tokdar et al. 2013). Flasks were incubated at 30°C on a rotary shaker rotating at 220 rpm for 96 h and dosed intermittently by 25 mg/L para hydroxy benzoic acid at 24 h followed by 5 ml of sucrose solution (30%) at 48 h and 72 h (Zhong et al. 2011; Ha et al. 2007). In the production medium we have replaced the carbon source from sucrose (50 g/L) to cane molasses (80 g/L) which has been reported earlier by other researchers (Yoshida et al. 1998).

Dry Cell weight (DCW) Measurement

Ten ml of broth was centrifuged at 12000 rpm for 20 min in a

Table 1: Different trials of protoplast fusion

Protocol	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
Washing buffer	TES buffer (0.01M,Tris HCL disodium EDTA 0.025M, Sucrose 0.6M, pH 8.0) 3 times wash	TES buffer, 3 times wash	0.5M sucrose containing 0.5mM disodium EDTA, single wash	0.5M sucrose containing 0.5mM disodium EDTA, 2 times wash and in 2nd wash time keep it for 20 min in washing buffer	0.5M sucrose containing 0.5mM disodium EDTA, single wash	0.5M sucrose containing 0.5mM disodium EDTA one wash with 0.5M sucrose and keep it for 1h in washing buffer
Resuspending buffer	0.6M Sucrose	0.6M Sucrose	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose
Lysozyme	1mg/ml	1mg/ml	1mg/ml	0.5mg/ml	1mg/ml, 0.25mg /ml	1mg/ml
Shaking	Water bath at 37ºC	Shaking intermittently	Shaking intermittently	Shaking intermittently	Shaking intermittently	Shaking intermittently
Adding protoplast samples	1:1 ratio	1:1 ratio	1:1 ratio	1:1 ratio	1:1 ratio	1:1 ratio
Wash (centrifugation at 2000 rpm for 10 min)	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose
Mixture made in 0.5M sucrose for fusion	PEG 30% (w/v) $CaCl_2$ 10 mM DMSO 15% (v/v)	PEG 40% (w/v) $CaCl_2$ 10 mM DMSO 5% (v/v)	PEG 40% (w/v) $CaCl_2$ 10 mM DMSO 15% (v/v)	PEG 40% (w/v) $CaCl_2$ 10 mM DMSO 5% (v/v)	PEG 40% (w/v) $CaCl_2$ 10 mM DMSO 5% (v/v)	PEG 40% (w/v) $CaCl_2$ 10 mM DMSO 5% (v/v)
Incubation period	5 min	10 min	10-15 min	10-15 min	10-15 min	10-15 min
Resuspending buffer	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose	0.5M Sucrose
Dilution plating	TSA, Regenerating medium (TSB + 0.5M Sucrose with 1.5% soft agar)	TSA, Regenerating medium (TSB + 0.5M Sucrose with 1.5% agar)	TSA, Regenerating medium (TSB + 0.5M Sucrose + $MgCl_2.6H_2O$ 5g/L+ $CaCl_2.2H_2O$ 3.7g/L+ 1.5% soft agar)	TSA, Regenerating medium (TSB + 0.5M Sucrose + $MgCl_2.6H_2O$ 5g/L+ $CaCl_2.2H_2O$ 3.7g/L+1.5% soft agar)	TSA, Regenerating medium (TSB + 0.5M Sucrose + 1.5% agar)	TSA, Regenerating medium (TSB + 0.5MSucrose + 1.5% agar)

pre-weighed centrifuge tube. The cell mass was quantified by drying at 60°C until a constant mass was obtained.

CoQ_{10} extraction method

CoQ_{10} extraction was performed in same way as described by Ranadive et al. 2011.

HPLC

The CoQ_{10} extracted from cell biomass was quantified on HPLC (Agilant Agilent 1100) using normal phase Kromasil silica column (250 mm x 4.6 mm, 5 µ particle size) and hexane: isopropyl alcohol (95:5) as mobile phase with a flow rate of 1ml/min. Detection was carried out at 273 nm (Tokdar et al. 2013).

Carbohydrate differentiation of mutant

Sterile plates containing the phenol red base agar were surface seeded with cell suspension made from mutant G-30, mutant P-87, fusant PF-P1 and allowed to dry for some time. The carbohydrate discs (Hi-Media) were placed and pressed gently on the surface of the plate at sufficient distance (2cm) from each other. Incubation was carried at 36±1.0º C for 18-48 h and results were recorded at 18-24 h and again at 48 h. We have done modification of the methods of biochemical characterization as described earlier by Urakami and Komagata, 1984.

AFLP

Isolation of DNA from test samples was performed using DNA isolation kit and the method has been developed by GeneOmbio Technologies Private Limited, Pune . Quality assessment of DNA was performed by agarose gel electrophoresis and using Qubit Fluorometer (Invitrogen). The method of gel electrophoresis has been modified as described previously by Chang and Chui, 1998. Restriction digestion was performed on 500 ng of the obtained DNA followed by ligation. The diluted ligation mixture was used for pre-amplification using AFLP® primers. For pre-selective amplification 4.0 µl of diluted DNA prepared by restriction-ligation, 0.5 µl of AFLP EcoRI preselective primer, 0.5 µl of AFLP MseI preselective primer and 15.0 µl of AFLP amplification core mix was combined in a PCR reaction tube (0.2 ml for the GeneAmp PCR System 9600). The samples were placed in a thermal cycler at ambient temperature. The PCR was carried out under the following conditions denaturation of duplex DNA at 94°C for 20 sec, primer annealing at 56°C for 30 sec and finally extension of the primer with a polymerase in the presence of dNTP at 72°C for 2 min using all ramp times as 0.01 sec on the Gene Amp PCR System 9600 and finally stored at 2-6°C. Ten µl of each reaction was loaded on a 1.5% agarose gel in 1X TBE buffer and run at 4V/cm for 3–4 h. The gel was stained with ethidium bromide. For selective amplification, templates were made by combining 10.0 µl pre selective amplification product and 190.0 µl TE buffer. It was then mixed thoroughly and spun down in a microcentifuge for 10 sec. Selective amplification

for EcoRI- and MseI-modified fragments was performed by combining extra space 1.5 µl diluted pre-selective amplification product, 0.5 µl MseI primer at 5 µM, 0.5 µl dye-labeled EcoRI primer at 1 µM and 7.5 µl AFLP core amplification Mix. The PCR was run using the thermal cycler parameters for selective amplification and then stored at 2-6°C. For loading samples in ABI3130 genetic analyzer (make Applied Biosystems, USA), 0.5 µl selective amplification products, 0.25 µl Gen Scan 500 Rox, 9.25 µl Hi-di Formamide pre thawed were prepared on the sequencer. The tubes were heated to 95°C for 3 min. Then they were quick chilled on ice. The samples were loaded in machine with required instrument protocol and the results were analyzed using Gene Mapper software. AFLP was done by modifying the methods as described previously by Aarts et al. 1998; Chang and Chui, 1998.

Genetic analysis

For estimation of Similarity–Coefficient (SC), the formula proposed by Nei and Li (1979) was used to generate computer algorithm and transformed into GD (Genetic Distance) Analysis profile. The SC was converted into GD using the equation GD=1-SC. Total number of alleles amplified was calculated using the formula: Nx+Ny-Nxy, where Nx, Ny and Nxy were total amplicons detected in mutant G-30, mutant P-87 and fusant PF-P1. (Nagee et al. 2003).

16S rRNA sequencing

The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$ + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1199 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.1 (Tamura et al. 2011).

UPGMA phylogenetic analysis

UPGMA approach implemented in the MEGA 5.1 program was employed for constructing phylogenetic relationships among sequences. The statistical reliability of the phylogenetic tree was tested by bootstrap analysis with 500 replications (Tamura et al. 2011).

Statistical analysis

For analyzing differences between two groups, Student's t-test was used based on PRISM-5 software. P values below 0.05 were considered statistically significant. The values in all graphs are an average of 3 trials. Unless stated otherwise, all error bars represent standard error of mean.

Accession Numbers of the Strains

The 16S ribosomal RNA gene sequence of mutant G-30, mutant P-87 and fusant PF-P1 was derived and deposited in NCBI GenBank (Accession number *KF676725*, *KF676726* and *KF676727* respectively). The fusant PF-P1 was deposited in Microbial Culture Collection (MCC) of National Center for Cell Sciences (NCCS), Pune, India under accession number *MCC2330*.

Results

Initially attempts were made to select a natural variant of *Paracoccus denitrificans* ATCC 19367 which can grow at higher concentration of cane molasses. In this process a single colony appeared on the agar plate containing 13% cane molasses. This colony was named as PdSr and was selected for further work. It was subjected to mutagenesis using γ irradiation. Around 99.99% reduction in viability was observed with the exposure of 250 γ irradiation. The selections of mutants were carried out based on the resistance to different antibiotics. Out of 30 antibiotics tested for sensitivity, the wild type strain was found to be sensitive towards 27 antibiotics having maximum sensitivity towards Neomycin, Vancomysin and Gentamycin. The γ irradiated suspension when plated on different antibiotics containing gradient plate, 93 no of Vancomycin resistant mutant colonies growing at a concentration above MIC level were obtained. Screening of these mutants resulted in over producing mutant G-30 that showed 1.06 fold improvement in specific CoQ_{10} content than the wild type strain.

Mutant G-30 was further subjected to NTG mutagenesis. Around 96.09% reductions in viability were observed with 400 µg/ml of NTG for 30 min of exposure. The NTG treated suspension was plated on to gradient plate containing Neomycin, Vancomycin and Gentamycin antibiotics respectively in order to isolate antibiotic resistant mutants. Subsequently 107 no of Gentamycin resistant mutants were obtained, which were screened for CoQ_{10} content. Mutant P-87 showed 1.25 fold improvement in specific CoQ_{10} content than wild type strain which was highest among all mutants. The sensitivity pattern clearly indicates that induced mutant G-30

Table 2: Proposed combinations of parental strains to carry out protoplast fusion.

Combination	Strains	Antibiotic marker
1	P-87	Gen^R,$Vanco^S$
	G-30	Gen^S,$Vanco^R$
2	PdSr	Gen^S,$Vanco^R$
	P-87NV4	Gen^R,$Vanco^S$
3	PdSr	Gen^S,$Vanco^R$
	P-87NV9	Gen^R,$Vanco^S$
4	PdSr	Gen^S,$Vanco^R$
	P-120	Gen^R,$Vanco^S$
5	G-30	Gen^S,$Vanco^R$
	P-87NV4	Gen^R,$Vanco^S$
6	G-30	Gen^S,$Vanco^R$
	P-87NV9	Gen^R,$Vanco^S$
7	G-30	Gen^S,$Vanco^R$
	P-120	Gen^R,$Vanco^S$

[R: Resistant; S: Sensitive; Vancomycin: 1.5µg/ml; Gentamycin: 3µg/ml]

acquired Vancomycin (1.5µg/ml) resistance where as induced mutant P-87 derived from mutant G-30 was found to be sensitive to Vancomycin (1.5µg/ml) but showed clear resistance to Gentamycin (3µg/ml). These two mutants showed improvement in specific CoQ_{10} content indicating the possibility of having desired mutations on the genes of biosynthetic pathway of CoQ_{10}. The property of antibiotic

resistance marker from these two strains could be utilized to carry out protoplast fusion between these strains.

Conformation of antibiotic resistant markers in mutant strains

The induced mutants generated from *Paracoccus denitrificans* ATCC 19367 having antibiotic resistance markers were used as parent strains for protoplast fusion experiments. The *Paracoccus denitrificans* 19367 and its derived mutant strains namely P-87, G-30, P-87NV4, P-87NV9, and P-120 having resistance to various antibiotics were generated and their respective antibiotic resistant markers were confirmed using antibiotic susceptibility test. These antibiotic markers were used as phenotypic markers for protoplast fusion. Depending upon the antibiotic sensitivity pattern and complementary markers, we have proposed different combinations of the strains to carry out fusion as shown in Table 2. Out of these different combinations, we have taken the first four combinations to carry out our present protoplast fusion study.

Table 3: Shake flask screening of strains.

Mutant/ Fusant	Origion	Titer (mg/L)	Specific CoQ10 content (mg/g of DCW)
Wild type strain	*Paracoccus denitrificans* ATCC 19367	25	0.8720
Pdsr	Sucrose resistant natural variant	25.5	0.8754
G-30	PdSr mutation using γ irradiation	27.2	0.9314
P-87	G-30 mutation using NTG	30.6	1.0921
PNV4G1	P-87NV4 +G-30	14.07	0.8029
PNV9G1	P-87NV9 +G-30	8.51	0.7938
SRNV9.10	PdSR+P-87NV9	16.51	0.8324
PF-P1	P-87+G-30	37.5	1.51
PF-P3	P-87+G-30	27.3	0.8791
PF-P5	P-87+G-30	28.7	0.9541
PGF7	P-87+G-30	21.69	0.8608

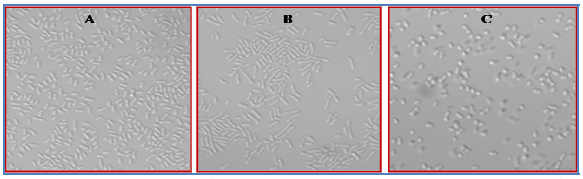

Figure 1: Laser Confocal Microscopic images of strains from seed growth (63X magnification) showing difference in morphology. [A: Morphology of mutant P-87, B: Morphology of mutant G-30, C: Morphology of fusant PF-P1]

Optimization of formation and regeneration of protoplasts

Protoplast formation was carried out as per different trials as shown in Table 1. Trial no 4 showed successful formation and regeneration of protoplasts. Microscopically the protoplasts were visualized as round shaped big cells whereas the parental strains were short rods in shape in their exponential phase of growth as shown in Figure 1. The fusants were selected on the regenerated medium plates having genetic markers of both the parental strains. Most of the fusants showed dual antibiotic resistant markers present in their respective parent strains. The generated fusants were screened by shake flask fermentation. Table 3 describes the titer and specific CoQ_{10} content obtained through shake flask screening of wild type, mutant and generated fusant strains.

Characterization of fusant PF-P1

CoQ_{10} over producing fusant PF-P1 was characterized using phenotypic and genotypic methods. Carbohydrates assimilation tests were carried out by carbohydrate differentiation discs method and results are shown in Table 4. The fusant PF-P1 showed differences in carbohydrate fermentation pattern than two parent strains with respect to arabinose, rhamnose, sorbitol and dulcitol. AFLP pattern analysis demonstrates that genotyping of mutant P-87, mutant G-30 and fusant PF-P1 could be accomplished on the basis of genomic variations with respect to polymorphic band pattern and also able to characterize intra-specific polymorphisms among them. Genetic analysis suggests that the three organisms showed genetic diversity with respect to AFLP patterns using four out of five primer pairs used for the present study. Out of 212 total numbers of fragments obtained 19 were polymorphic means that bacterial strains analyzed by AFLP show 8.96% polymorphic fragment pattern as shown in Table 5.

Table 4: Carbohydrate disc test using phenol red base agar medium

Carbohydrate	Parents Mutant G-30	Mutant P-87	Fusant PF-P1
Dextrose	++	+	++
Arabinose	++	+	-
Xylose	++	-	++
Sucrose	+	+	++
Melibiose	++	-	-
Salicin	+	-	-
Maltose	++	+	++
Fructose	+	+	++
Mannitol	-	+	++
Galactose	++	+	++
Terhalose	+	+	++
Rhamnose	-	-	++
Sorbitol	+	+	-
Lactose	++	+	++
Mannose	+	+	++
Cellobiose	++	-	++
Adonitol	-	+	-
Dulcitol	-	-	+
Inulin	-	-	-

[++ Fermenting, – Not fermenting, + Slow fermenting]

16SrRNA sequence analysis

The phylogenetic tree was prepared by comparing the conserved regions of 16S rRNA from PF-P1 with 16S rRNA gene sequence from 10 other genera along with mutant G-30 and mutant P-87 were shown in Figure 2. The analysis revealed that PF-P1 is having about 99% similarity to *Paracoccus denitrificans* and other *Paracoccus* sp and also acquired eight nucleotide base pair mutation on conserved region which depicts the evidence of stable mutation.

Phylogenetic analysis using UPGMA method

The phylogenetic tree indicated that the fusant PF-P1 has a distinct clade than the *Paracoccus denitrificans* strains. The analysis suggests that the fusant exhibits 16S rRNA gene sequence different from *Paracoccus denitrificans* ATCC 19367 strain. Distance matrix analysis using MEGA 5.1 showed that the PF-P1 sequence has genetic distance of 0.007

from other reported strains of *Paracoccus* sp. as well as the parent *Paracoccus denitrificans* ATCC 19367.

Discussion

The protoplast fusion in *Paracoccus denitrificans* strain has not been reported till date and hence a strategy has been undertaken to investigate the effect of genome shuffling on CoQ_{10} production. The protocol to carry out protoplast fusion was designed by modification of the various methods for protoplast fusion in gram positive as well as gram negative strains (Gong et al., 2009; Zhang et al. 2002). In the course of fusion, it was observed that the cells at the exponential phase on TSB medium having an optical density (OD) of about 1.0 had best protoplast. Under this condition, the clumps formation was arrested after addition of lysozyme. The mutant strains were found to be very sensitive to the lysozyme concentration at 1 mg/ml, as it showed immediate clumps formation. Hence the lower concentration of lysozyme was tried to avoid clump formation. Optimum concentration of lysozyme was found to be 0.5 mg/ml for ideal protoplast formation (Weiss et al. 1976). Before adding lysozyme, the pre-treatment with washing buffer to remove the LPS layer is essential. The protoplasts from two different phenotypes having different genetic markers, generated under optimal conditions were fused using appropriate concentration of PEG and time for fusion. Recombination between the genomes of these different strains can lead to strains that grow on regeneration medium in the presence of both Gentamycin (3µg/ml) and Vancomycin (1.5µg/ml) markers. Out of different trials carried out, PEG6000 of 40 % (^{w}/v) and fusion time of 10-15 min was found to be satisfactory to generate fusants. It is very essential to regenerate protoplasts on a suitable agarified medium. The medium must support the nutrients and salts for formation of the LPS and peptidoglycan layer without causing the lysis of the protoplast. The initial regeneration medium containing TSB with 0.5M sucrose enriched by the addition of $MgCl_2.6H_2O$ (5 g/L) and $CaCl_2.2H_2O$ (3.7g/L), showed 20 fold improvement in regeneration frequency. This protocol gave consistently high regeneration frequency above 65% and was used for subsequent protoplast fusion experiments. The ideal fusants were selected on the regenerated medium plates having genetic markers of both the parental strains. Most of the fusants showed dual antibiotic resistant markers present in their respective parent strains. The two fusants namely PF-P3 and PF-P5 showed loss of Gentamycin resistance marker which was present in their respective parent strains. This might be due to the genetic random recombination in the Gentamycin coding sequence leading to the insertional inactivation of this gene. The fusants that retained both the resistant markers from parent strains did not show improvement in CoQ_{10} titer except fusant PF-P1. The fusant PF-P1showed 1.62 folds and 1.38 folds improvements in specific CoQ_{10} content than both parents i.e. mutant G-30 and mutant P-87 respectively. Mutant G-30 and mutant P-87 were found to produce specific CoQ_{10} content of 0.9314 mg/g of DCW and 1.0921mg/g of DCW respectively where as fusant PF-P1 was found to produce 1.51 mg/g of DCW at same

Table 5: Level of genetic information generated by each primer pair using AFLP.

Primer Combination	Wild type strain	Samples and total number of bands obtained after fragment analysis			Total number of fragments detected by each primer combination (a)	No of common bands	No of polymorphic bands (b)	% polymorphism obtained using primer combination (b/a)*100%
		Mutant G-30	Mutant P-87	Fusant PF-P1	Total			
EcoR1-FAM-A/Msel-T	9	5	8	5	27	5	4	14.81%
EcoR1-FAM-A/Msel-G	6	5	6	9	26	5	3	12%
EcoR1-FAM-0/Msel-A	6	7	7	6	26	6	1	3.85%
EcoR1-FAM-0/Msel-C	23	15	23	23	84	15	8	10%
EcoR1-FAM-0/Msel-T	12	12	13	12	49	11	3	6%
Total	56	55	57	44	212	42	19	8.96%

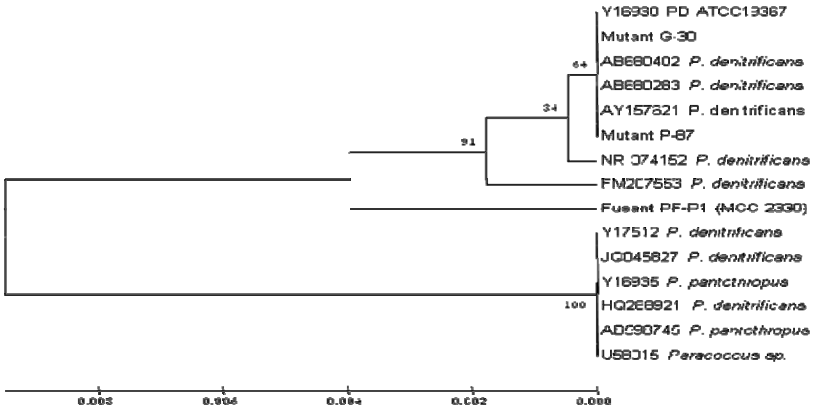

Figure 2: Phylogenetic analysis of fusant PF-P1 using 16S rRNA sequence analysis. [The optimal tree with the sum of branch length=0.02519610 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1091 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.1]

experimental conditions. In comparison with wild type strain *Paracoccus denitrificans* ATCC 19367, fusant PF-P1 showed 1.73 folds improvements in specific CoQ_{10} content, as the wild type strain was found to produce 0.8720 mg/g of DCW.

The genetic stability of fusant is an important feature which determines the potential of the strains for commercial production. Often the derived fusants tend to lose genetic markers by reversion thereby decreasing the productivity of the desired metabolite. In order to check the genetic stability of the selected overproducing fusant PF-P1 along with the two parents i.e. mutant G-30 and mutant P-87 were sub-cultured to make subsequent generations and

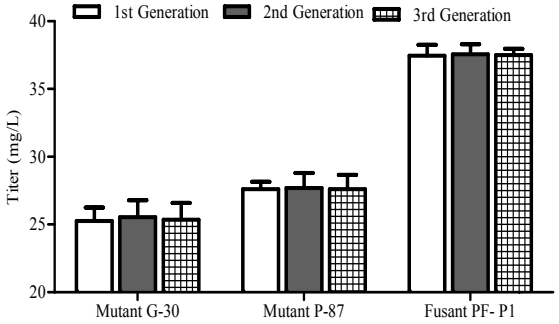

Figure 3: Comparison of CoQ10 titer of mutant G-30, mutant P-87 and fusant PF-P1 in subsequent three generations. [For statistical analysis student's t-test were used based on PRISM-5 software. The values in the graph are an average of 3 trials. All error bars represent standard error of mean.]

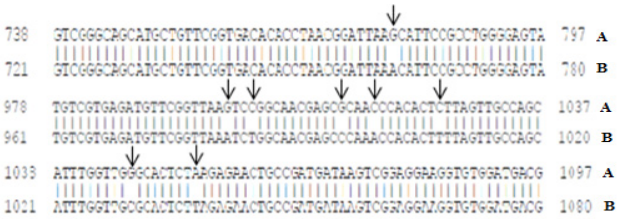

Figure 4: Number of nucleotide base pair mutation occurred in fusant PF-P1. [A: Paracoccus denitrificans ATCC 19367, B: Fusant PF-P1, Bottom arrows indicates the base pair mutation of the nucleotide sequence in the conserved region.]

tested for the CoQ_{10} productivity. All three strains have been tested for the CoQ_{10} productivity in subsequent three generations as shown in Figure 3. For statistical analysis student's t-test was used based on PRISM-5 software. The values in the graph are an average of 3 trials. All error bars represent standard error of mean. There was no significant difference in CoQ_{10} productivity observed in subsequent three generations of the strains indicating stable characteristics of these strains till third generations.

The AFLP pattern obtained for two parent strains and fusant PF-P1 suggest close relation of each isolate with each other due to number of common fragments obtained using different primer combinations. The effectiveness of this method in strain discrimination is seen in the separation of three different organisms. For strains of the same species, the number of polymorphisms between strains ranged from one to eight from a single primer pair. Selective amplification using primer pair EcoRI-FAM-0/MseI-C provided high resolution data amongst all five primers. Total 212 markers were amplified in three organisms of which 42 markers were common to all strains and 19 markers showed polymorphic appearance in one or the other strains. These results suggest the presence of intra-specific polymorphism amongst the strains.

Cluster analysis of the type strains of *Paracoccus denitrificans* was performed using the results from five primer pairs. It reveals that mutant P-87 has smaller genetic distance with type strain (0.131) whereas mutant G-30 shows greater genetic distance with type strain (0.372). Fusant PF-P1 has genetic distance of 0.197 with the type strain *Paracoccus denitrificans* ATCC19367. The 16S rRNA gene sequences of fusant PF-P1 acquired eight nucleotide base pair mutation on conserved region as shown in Figure 4. This evidence clearly indicates that after having stable mutation in conserved region, fusant PF-P1 showed 99% similarity to *Paracoccus denitrificans* and other *Paracoccus* spp and did not revert back to the original strain.

Conclusion

We have successfully demonstrated the methods of standardizing protoplast generation, regeneration and subsequent fusion. Upon screening of fusants using standardized condition one fusant namely PF-P1 showed improvement in CoQ_{10} production as well as in specific CoQ_{10} content. Biochemical and molecular characterization of fusant PF-P1 showed difference than wild type strain. Finally the fusant PF-P1 was identified by 16S rRNA gene sequence analysis where it showed 99% similarity to *Paracoccus denitrificans* and other *Paracoccus* spp along with eight nucleotide base pair mutation on conserved region. In conclusion, the generated fusant PF-P1 is a stable novel fusant strain with enhanced capacity of CoQ_{10} production. The further work on fermentation optimization using this novel fusant strain PF-P1 can lead to a successful process for production of CoQ_{10}.

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