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Genetic manipulation of some bacterial strains for improvement of PUFA production

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Abstract

Polyunsaturated Fatty acids (PUFA), classified as omega-3 and omega-6 families, are essential fatty acids as these cannot be synthesized de-novo and must be supplied in the diet. A cursory look at literature demonstrates the positive role of ω-3 fatty acids in alleviating variety of life style and life threatening diseases and health conditions. In the present study 3 bacterial strains were subjected to random mutagenesis using UV irradiation and MMS (Methylmethanesulfonate) treatment for improvement of PUFA production. Variety of mutants obtained after exposure to mutagens with varied time or concentration demonstrated that random mutagenesis can be a simple, quick and promising technique for obtaining mutants either with improved or modified PUFA content as judged by the GC analysis. Fourteen mutants with interesting PUFA composition or increased amount of PUFA production were identified. Mutants B. sps. FPZSP13/UV/1, B. sps. FPZSP13/UV/2, H. sps.QY113/UV/2, H. sps.QY113/MMS/1, B.t 407/UV/1 and B.t 407/MMS/2 produced significantly higher amounts of a single or variety of PUFA like Linoleic acid, Alpha-linolenic acid, Eicosatrienoic acid, etc. Further characterization of such mutants revealed that mutantsH. sps.QY113/UV/2 and B.t 407/MMS/2 also have improved biomass production. Further mutagenesis program with these mutants coupled with media and process optimization can be employed to develop few of them into commercially promising mutants to produce either single PUFA or mixture of useful PUFA.

Keywords- PUFA, ω-3 fatty acids, ω-6 fatty acids, Random Mutagenesis, UV, MMS, GC.

I. INTRODUCTION

Polyunsaturated fatty acids (PUFA) belong to the class of simple lipids and are fatty acids with two or more double bonds. There are two main families of PUFA: n-3 and n-6. Some of them are Linoleic acid (LA) 18:2 ω-6, Alpha-Linolenic acid (ALA) 18:3 ω-3, Eicosapentaenoic acid (EPA) 20:5 ω-3, Docosahexaenoic acid (DHA) 22:6 ω-3, Arachidonic acid (AA) 20:4 ω-6, etc.[1].PUFA are generally known as essential fatty acids as animals, including humans are not capable of efficiently building these long chain omega fatty acids, and must acquire them from their diets. It is well recognized that dietary intake of n-3 PUFA has profound benefits on normal health and prevention of chronic disease states [2].

Of late, attention has been focused on bacterial production of long chain and valuable PUFA for two main reasons. Firstly, bacteria can be easily cultivated in simple media which makes them a prime candidate for industrial production. Secondly, it is possible to genetically manipulate bacteria in order to increase production of a metabolite of interest. Also, it may be possible to command a premium in market if PUFA are obtained from microbial sources rather than plant and animal sources as these are limited in nature and are also needed as primary sources of food [3]. The quality and supply of Single Cell Oils (SCO) by bacterial sources can be closely controlled and continuous production of PUFA can be achieved in order to meet ever increasing demand [1].

Mutagenesis is routinely practiced in the pharmaceutical industry to quickly induce changes in the DNA of microorganisms in order to increase their productivity. Because natural mutagenic process is extremely slow, physical and chemical mutagens are used to introduce random mutations in the population thus accelerating the mutation process[4].Thus, mutagenesis is well established and an incredibly powerful tool for genetic strain improvement, which along with media and process optimization is the core of process development activity for any commercial fermentation process [5,6,7,8,9].

Recent advances in the field of molecular biology and microbial genetics have revealed new possibilities to bring about yield. A collection of promising access points for genetic and metabolic engineering are now available concerning synthesis, turnover and control of fatty acids [10]. Developing high lipid accumulating microorganisms or engineered strains for specific PUFA production is a potentially promising area of research [11]. Despite the advent of molecular biology and other rational screening techniques, classical random mutation and screening still plays a large role in improving industrial strains and have driven down the cost of production of a wide range of fermentation products. This has been possible because it is a cheap and reliable method that is simple to apply and use even when the strain under study is not genetically well characterized. Besides the mutants derived from classical mutagenesis methods do not require any special handling or permissions from regulatory authorities [12].

In wild type or typical cultures a limited increase in production of microbial metabolites like SCO (Single Cell Oil), PUFA included, can be achieved by process and media optimization. Further increase can be achieved only by carrying out genetic manipulations. In practice, strain improvement goes hand-in-hand with medium and process improvement, and the high titres seen in many fermentation processes today are the result of both being employed synergistically [13, 14, 15].

Use of random mutagenesis, coupled with application of intelligent screening methods for selection of desired mutations has proved to be very cost effective and time saving strategy for the fermentation industry especially for production of antibiotics and secondary metabolites [5].Natural rate of spontaneous mutation is very low and hence practically of no use for introducing quick changes. On the other hand random mutagenesis considerably accelerates the rate of mutation through the action of certain mutagens like Ultra-Violet radiations (U.V.), Gamma rays, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), Ethylmethanesulphonate (EMS), Methylmethanesulphonate (MMS), etc[7,12,16].

Oleaginous microorganisms of industrial interest have been successfully genetically improved by random mutagenesis by many researchers [6,9,14,17,18]. The present study was undertaken to establish kill curves and also to isolate mutants with enhanced PUFA production with increased titre or decrease in other undesired fatty acids.

II. MATERIALS AND METHODS

A. CULTURES

Three cultures namely*B.*sps. FPZSP13, *Halomonas* sps. QY113and *B. thuringiensis* Bt407 were mutated. These cultures were obtained from the salt water fish *Sardina longiceps*, Arabian Sea and a fresh water river Pindhari, Uttaranchal, India respectively, as reported previously [19,20].

B. MUTAGENS

Mutagenesis was carried out by means of short wave (254nm) U.V. radiation (15W UV lamp, GE). Methyl Methane Sulphonate (MMS) was used as the chemical mutagen and was procured from Himedia® Laboratories Pvt.Ltd., India.

C.GROWING CULTURE FOR MUTAGENESIS

A loopful of actively growing culture from a slant was inoculated into 50mlSemi synthetic minimal medium contained in a 250ml Erlenmeyer flask[20].Briefly, the flasks were incubated at 25^{o}C for 24hr on an orbital shaker at 100 rpm (stroke 25mm), (Orbitek, Scigenics Biotech, India). Salt water Semi synthetic minimal medium was used for *B.*sps. FPZSP13 and *H.*sps.QY113while Semi synthetic minimal medium prepared in distilled water was used for culturing *B.t*407.

D.PREPARATION OF CULTURE AND U.V. MUTAGENESIS

O.D of the culture broth at 20-24 hr age was read at 530nm on a colorimeter (Equiptronics EQ-650, India) and diluted to 0.4 using sterile saline. The viable cell count at 0.4 OD was determined by plating and was found to be $4.5\pm0.2\times10^{8}$ CFU/ ml for B. sps. FPZSP13, where as the viable count was $3.6\pm0.8\times10^{10}$ CFU/ ml for *Halomonas* sps. QY113 and $3.1\pm0.3\times10^{8}$ CFU/ ml for *B. thuringiensis* Bt 407.

Five ml of O.D adjusted culture suspension was transferred to a sterile Petri dish and the plate was positioned inside the UV Transilluminator box at a distance of 18cm from the UV light source. The cell suspension was exposed for 30, 60, 90 and 120 seconds by opening the lid of the plate. The plates were gently agitated intermittently for uniform exposure of cells to U.V radiation [9].

One ml cell suspension was withdrawn after exposure, serially diluted and 100µl of dilution was plated in triplicate on Semi synthetic minimal medium salt agar. Semi synthetic minimal medium distilled water agar was used for plating UV treated culture suspensions of *B.t*407. The plates were then incubated at 25°C up to 4 days and observed at 24hr intervals for the appearance of colonies.

E.PREPARATION OF CULTURE AND MMS MUTAGENESIS

One ml of actively growing culture (O.D: 0.4 at A_{530nm})was added to 4ml of Semi synthetic minimal medium contained in 20ml capacity tubes. Methyl Methane Sulphonate (MMS) was added to achieve concentrations of 10, 25, 50, 100 µl/ml of culture medium. The mutagen containing flasks were incubated at 25^{o}C, on an orbital shaker, stroke 25mm (Orbitek, Scigenics Biotech, India) set at 120 rpm for 120 min [21].One ml of growth from each of the flasks was centrifuged and the pellet was washed twice with saline to remove the adhering MMS and resuspended in 1 ml saline and serial dilution plating was carried out to obtain isolated colonies. The plates were incubated at 25°C up to 4 days and observed at 24hr intervals for the appearance of colonies.

F.SCREENING OF MUTANTS

Mutagenic procedures can be standardized in terms of type of mutagen, its dose and the time of exposure. The mutagenesis program is not restricted to construction of a kill curve but to find out the mutagenic conditions that result in production of desirable mutations. Devising efficient screening methods in order to identify from amongst the survivors, mutants of interest is quite a challenging task[15].

Due to lack of any rational screening method, survivors from exposure to mutagens were randomly selected. Some morphological variants were also retained. The selected colonies were transferred to agar slopes and allowed to grow luxuriously (48-72 hr). The Gram nature and morphology was noted under light microscope (1000X magnification). Next, lipid producing ability was checked qualitatively by Sudan Black B staining as per the method reported by Patnayak and Sree [17].

Retained mutants were further evaluated for lipid accumulation and PUFA profile in shake flask fermentation as per methods described by Masurkar and Vakil[22].The mutants were also checked for normal and vigorous growth by determining dry cell weight (DCW) as random mutagenesis can induce several mutations some of which can negatively affect the vigour and growth characteristics [5,15].

G.PRESERVATION OF MUTANTS

The mutants were preserved on slants and stored at 4^{o}C as well as in 40% glycerol stock which was held at -20^{o}C [23].

H.SHAKE FLASK FERMENTATION

Liquid Semi-synthetic minimal medium was used for all shake flask studies. All experiments were performed in 250ml Erlenmeyer flasks containing 50mlof semi-synthetic minimal medium with carbon to nitrogen (C/N) ratio adjusted to 40:1. The pH of the medium was 5.8 for salt water containing medium and 5.3 for the fresh water medium. The preparation of inoculum and subsequent measurement of growth by Dry Cell Weight (DCW) was done as described by Masurkar and Vakil [22].Primary screening was performed by Sudan Black B staining as per Masurkar et al. [19]. Finally lipid extraction, quantification followed by derivatization to FAMEs to be analyzed by GC was done as reported previously [20].

Chromatographic comparison with authentic FAME standards from Supelco was used for identifying the FAME derived from isolates. The presence of PUFA if any and their relative quantity w.r.t other fatty acids was estimated from the GC peak area percentage.

Shake flask experiments were performed in triplicate to confirm the reproducibility of results for the promising mutants. Mutants displaying PUFA production, lipid accumulation and superior growth were retained for further characterization and scale up studies.

III. RESULTS AND DISCUSSION

The kill curves established for UV and MMS mutagenesis for the *B.* Sps.FPZSP13 is shown in Figure No.1.

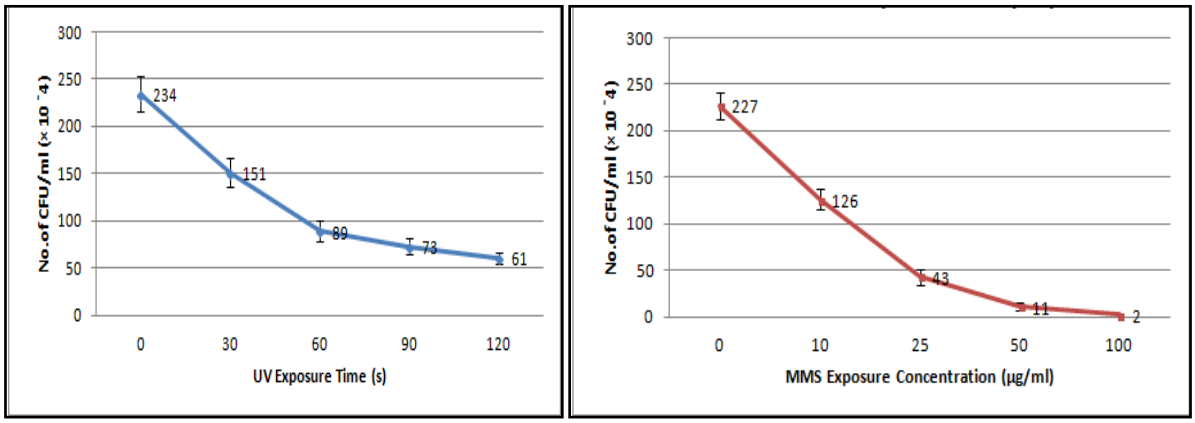


Figure 1. ***UVKill curve (I) and MMS kill Curve (II) for B.*** **sps.** ***FPZSP13.***

As may be noted from the trend of CFU in the Figure No.1.I, significant decrease was observed up to 60seconds of exposure to UV after which the reduction in number of survivors was not noteworthy. Highest kill of 73.93% was observed at 120s of UV exposure. Sixty seconds can be considered as the reasonable exposure time for UV mutagenesis for parent culture *B.* sps. FPZSP13.Sixteenputative mutant colonies were selected from amongst the survivors of UV mutation treatment. Another kill curve was established when the parental *B.* sps. FPZSP13 culture was exposed to mutagen MMS (Figure No.1.II). A sharper decrease in the viability was observed up to 25 µl/ml of concentration of MMS after which the reduction in number of survivors was gradual. A highest kill of 99.12%was observed for the parent *B.* sps. FPZSP13 at MMS concentration of 100µl/ml. Thus the reasonable dosage for MMS mutagenesis can be considered to be 25 µl/ml. Sixteen putative mutant colonies were retained from the 4 MMS concentrations used for the mutation treatment.

Figure No.2 shows the kill curves for parental culture *H.* sps.QY113 exposed to UV and MMS mutagenesis. A time of exposure dependent decrease in the survivors post UV irradiation was observed right up to 120 seconds of exposure. A maximum of 96.34% kill was observed at 120 seconds of UV exposure.

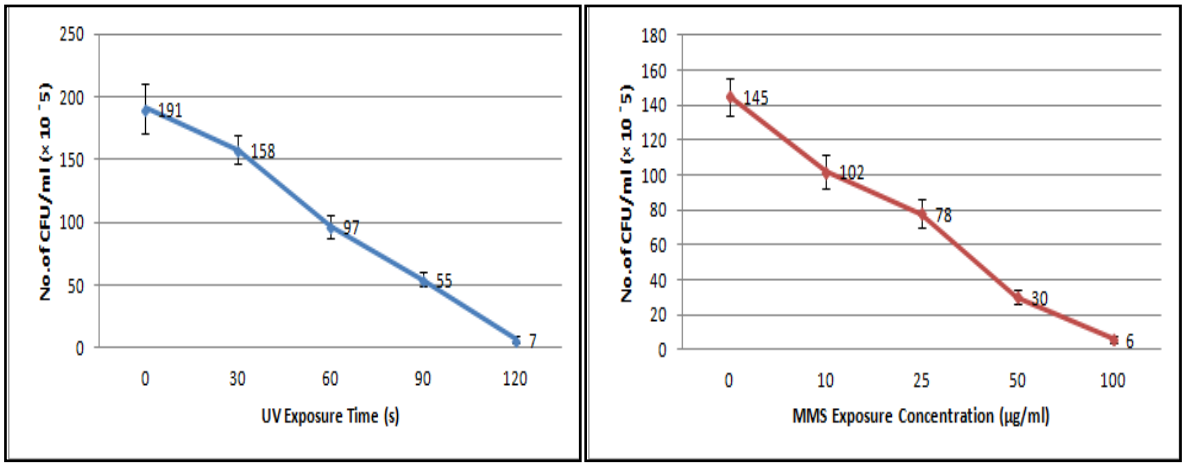


***Figure2.UV Kill curve (I) and MMS kill Curve (II) for H.* sps.*QY113*.**

Three putative mutant colonies each from treatment time of 60 and 90swere retained for further evaluation. Figure No. 2.IIshows the kill curve for parent *H.* sps.QY113 when it was exposed to various concentrations of another mutagen, MMS. A steady decrease in the CFU was observed upto100 µl/ml of MMS.A maximum of 95.86% kill was observed at a concentration 100 µl/ml of MMS. Six of the putative mutants retained from this mutagenic treatment included 4 derived from 10 µl/ml concentration, 01 colony from 50 µl/ml concentration and 01 more colony from the100 µl/ml concentration of MMS.

Figure No. 3.Ishows the kill curve plot for *B. t* 407, the third culture that was mutated by exposure to UV light. Approximately 76.27%of cells of the parent *B. t* 407 were killed by 120 seconds of exposure to the mutagen. A steady decrease in the CFU was observed upto 90 seconds of exposure. Further increasing the exposure time did not result in much reduction in no. of survivors**.** A total of 6 surviving colonies were retained as potentially useful mutants where one mutant colony was derived from 30 seconds of exposure, another mutant colony came from 60 seconds exposure, and 02 each were selected from 90 seconds and120 seconds exposure time.

Figure No. 3.II shows the kill curve for the third parental culture-*B. t* 407that was exposed to MMS.A very high % about 95.41%of exposed cells of the parent were killed at a concentration 100 µl/ml of MMS. Nine of the putative PUFA producing mutants that were selected from this trial

of which 01 mutant was obtained from 25 µl/ml of MMS concentration, 02 were derived from 50 µl/ml MMS concentration and remaining 06 mutants from 100 µl/ml of MMS.

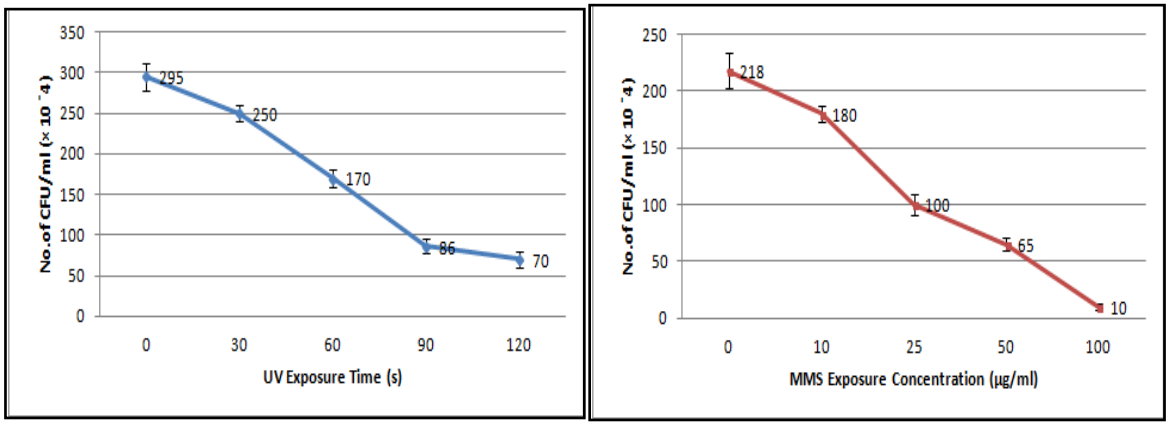


Figure 3. UV Kill curve (I) and MMS kill Curve (II) for B. t407.

All the 59 colonies that were retained were transferred to agar slants, allowed to grow for 48-72 hr and then they were grown along with 3 parental cultures, in the shake flask as per the procedure described in Materials and Methods and the contents of the flasks were analyzed for various parameters. The first criterion for selection was the ability to grow in the liquid medium followed by Sudan Black B staining for lipid production. Only those growing reasonably well and testing positive for Sudan Black B staining were processed further for determination of PUFA and composition of PUFA by GC analysis. Results for 14 promising mutants and 03 parental cultures (for comparison) are shown in Table No.1. Some representative chromatograms are attached to show types of PUFA produced.

Table 1. Selection of mutants and their PUFA production profile.

Parent/Mutant Culture	Exposure to Mutagen	Type of PUFA	% of total PUFA	GC Chromatogram
B. sps. *FPZSP13 (Parent)*	NA	LA	3.55	Figure 4
Mutants				
B. sps. FPZSP13/UV/5	U.V-90 Sec	LA, ALA, AA	42.10	
B. sps.FPZSP13/UV/14	U.V-90 Sec	LA	12.26	
*B.*sps. FPZSP13/MMS/14	MMS-25 µl/ml	LA	12.51	
*B.*sps. FPZSP13/MMS/15	MMS-25 µl/ml	GLA, LINA	7.61	-
H. sps.*QY113 (Parent)*	NA	LA, ETA	3.36	Figure 5
Mutants				
H. sps.QY113/UV/1	U.V-60 sec	LA, EDA, ETA	16.43	
H. sps.QY113/UV/7	U.V-90 sec	LA,ALA,EDA,	27.89	

		ETA		
H. sps.QY113/UV/9	U.V-90 sec	LA, ETA	9.66	
H. sps.QY113/MMS/1	MMS-10 µl/ml	LA	27.16	
H. sps.QY113/MMS/2	MMS-10 µl/ml	LA, ALA	17.51	-
B. t 407 (Parent)	NA	LA	2.80	Figure 6
Mutants				
B. t 407/UV/5	U.V-90 sec	LINA	61.96	
B. t 407/MMS/6	MMS-100 µl/ml	LA,LINA, ALA, EDA, EPA	52.97	
B. t 407/UV/9	U.V-120 sec	LA, ETA, AA	5.17	-
B. t 407/UV/12	U.V-120 sec	LA	23.11	-
B. t 407/MMS/2	MMS-25 µl/ml	LA,ALA, EDA, ETA, AA, EPA	31.67	-

Key: LA=Linoleic acid ALA=Alpha Linolenic Acid, EDA=Eicosadienoic acid, EPA=Eicosapentaenoic Acid , ETA=Eicosatrienoic acid., GLA= Gamma Linolenic Acid, LINA = Linoleiadic acid, UV mutagenesis was carried out by varying the exposure time in seconds whereas MMS mutagenesis was carried out with varying MMS concentration with fixed exposure time of 120 min.

All the 3 parents make LA as was reported in previous publications related to screening and optimization of cultures for production of PUFA [19, 20, 22]. Subsequent work presented here show that the LA made by them is in low % quantities – 3.55, 3.36 and 2.80 (Table I, GC Chromatogram Figure No.4.I, 5.I, 6.I) as against 4 mutants which make 3-9 fold LA, for instance -*B.* sps.FPZSP13/UV/14 (12.26%, GC chromatogram Figure No. 4.IV), *B.*sps. FPZSP13/MMS/14 (12.51%, Table 1.), *B. t* 407/UV/12 (23.11%, Table 1.) and *H.* sps.QY113/MMS/1(27.16%, GC chromatogram Figure No. 5.II).The increase in LA production can be considered dramatic. Two main fatty acids essential in the diet are linoleic (or omega-6) fatty acid and alpha-linolenic (or omega-3) acid. Both of them are polyunsaturated fatty acid, that possess two or more double bonds and lack several hydrogen atoms that are found in saturated fatty acids.

*B.*sps. FPZSP13/MMS/15 (7.61% No ref chromatogram), *B. t* 407/UV/5 (61.96 %, GC Figure No. 6.II), *B. t* 407/MMS/6 (52.97%, GC chromatogram Figure No. 6.III) produced Linoleiadic acid, which none of the parent cultures produced. This demonstrated the effect of mutation which has altered the metabolic pathway of PUFA synthesis forcing mutants to produce structurally related compound that too in very good quantity. Linolelaidic acid is non-conjugated omega-6 trans fatty acid (TFA)and is a geometric isomer of linoleic acid[24].Similarly there is a mutant *H.* sps.QY113/UV/9overproducing the 2 PUFA- LA and ETA as a mixture and in 3 times more quantity than the parental culture- 9.66 % Vs. 3.36(GC Chromatogram Figure No. 5.IIIand 5.I).*B. t* 407/MMS/6 is the highest producer (52.97% GC Figure No. 6.III) of the mixture of LA, LINA, ALA, EDA, EPA amongst the three mutants that produce the mixture of 3 compounds i.e. mutant *B.* sps. FPZSP13/UV/5 producing mixture of LA, ALA, AA (42.10 %, GC chromatogram Figure No. 4.III)and *H.* sps.QY113/UV/7producing mixture of LA,ALA,EDA,ETA (27.89% GC Figure 5.IV). Mutant *B. t* 407/UV/9 produced long chain PUFA like ETA and AA which the parent culture did not produce.

A shift to higher carbon number fatty acids is seen in all the mutant lipid profiles depicted in the chromatograms labelled as Figure No. 4, 5 and 6 where the PUFA profiles of mutants are compared with their parental culture. As can be seen from the 3 GC chromatograms shown below, mutant *B.* sps. FPZSP13/UV/5, *H.* sps.QY113/UV/1 and *B. t* 407/MMS/6 all produced several short chain fatty acids as well produced high amounts of PUFA. On the other hand, mutant No. *B. t* 407/UV/12 is capable of producing 23% and *H.* sps.QY113/MMS/1 is capable of producing 27% of a single type of PUFA i.e. Linoleic Acid, thus can be a good candidate to develop it for the production of LA (Table 1).

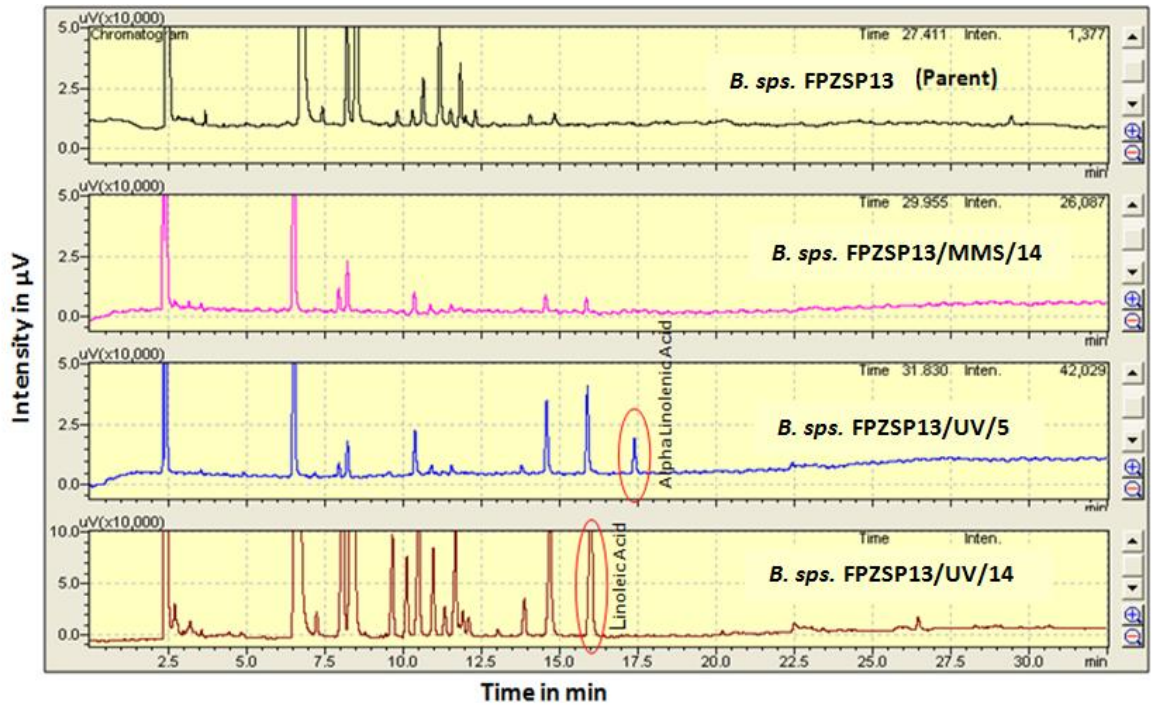


Figure 4. Gas chromatograms showing the lipid profile for parent B.* sps. *FPZSP13 and its mutants.

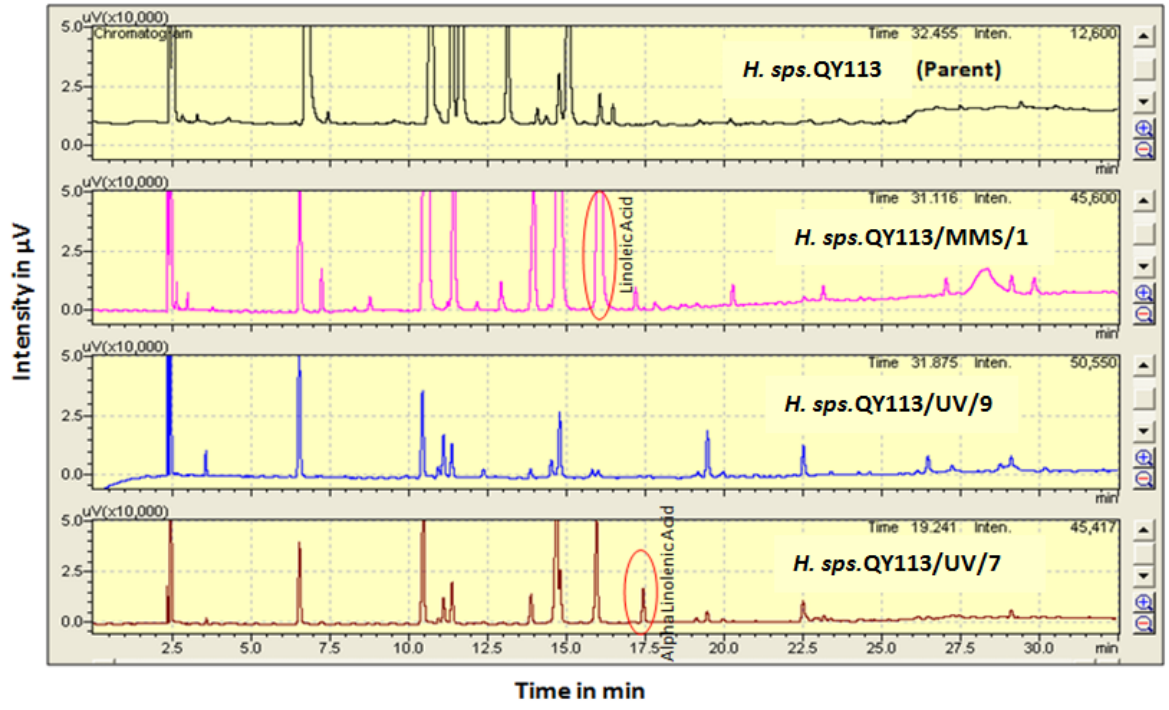


Figure 5. Gas chromatograms showing the lipid profile for parent H.* sps.*QY113 and its mutants.

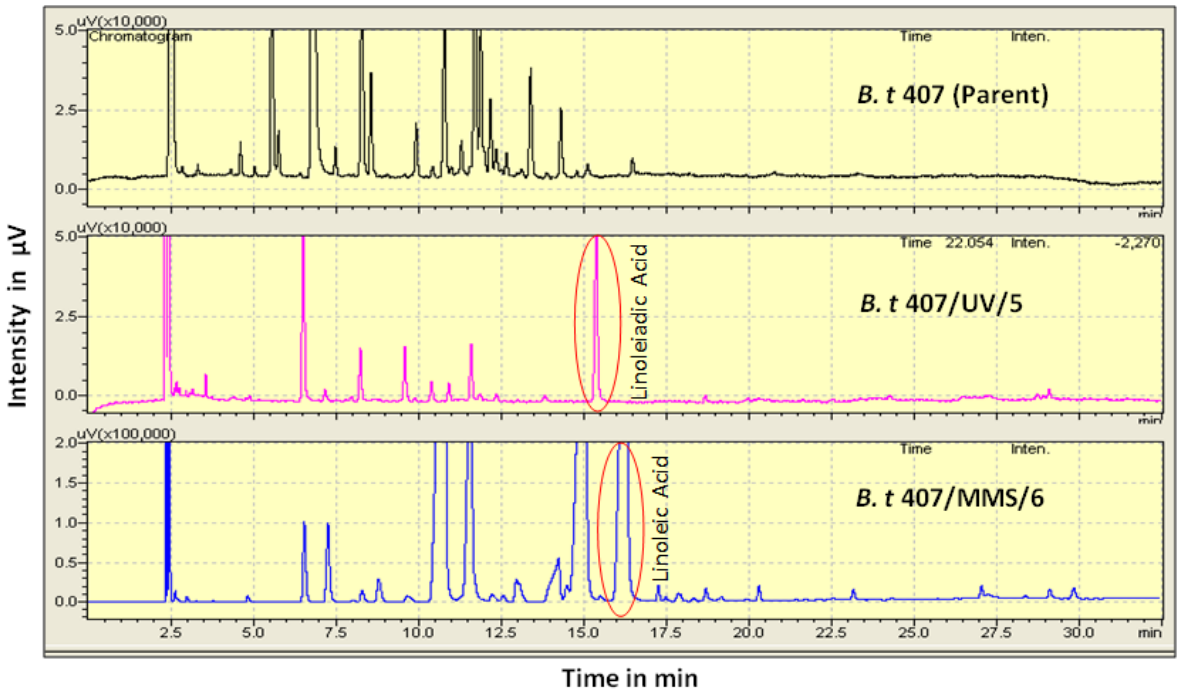
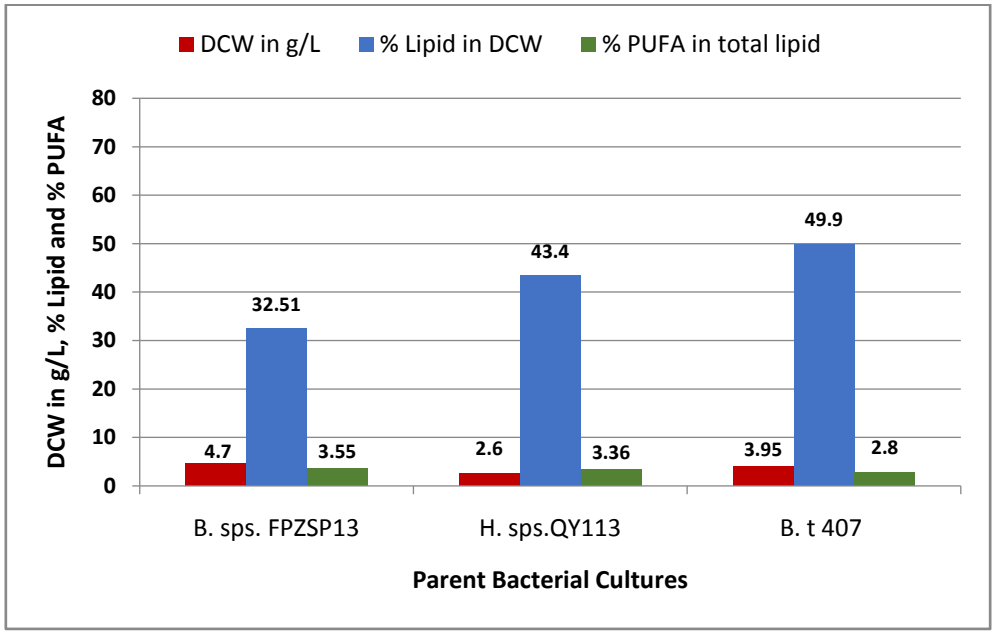


Figure 6. Gas chromatograms showing the lipid profile for parent B. t 407 and its mutants.

Particular attention has to be paid to the proportions of PUFA in the diet, which are the factors that determine the apparently unique balance of tissue n-3 and n-6 fatty acids and eicosanoids decisive for human health and well being [25]. A combination of beneficial PUFA was seen to be produced by mutants like B. sps. FPZSP13/UV/5, *H. sps*.QY113/UV/7, *B. t* 407/UV/9 and *B. t* 407/MMS/2,*B. t* 407/MMS/6. These have prospects for use in the production of mixtures of PUFA. The fact that some of the select mutants could produce high amounts of MUFA like myristoleic acid and oleic acid, is an interesting finding (Data not shown). This puts forward the possibility of these microbes for the production of valuable MUFA-PUFA combinations.

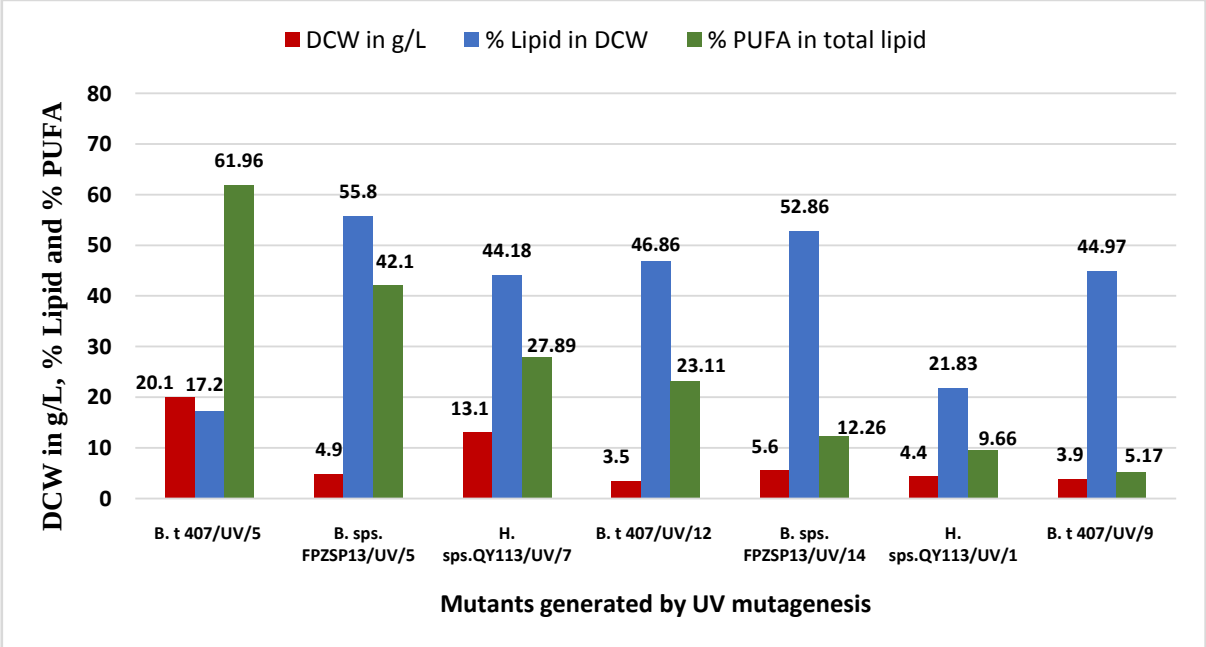
From all the mutants discussed so far 14 mutants having interesting PUFA content or PUFA components along with the 3 parental cultures were grown again in shake flask, now in triplicateand their biomass production, lipid production as well as the PUFA production was assessed at the harvest time by pooling the three flasks before analysis. The mutant *H.* sps. QY113/UV/9 did not grow well and hence was not analyzed further. Results for the remaining 13 mutants are presented as Figure No. 7for parental cultures, Figure No. 8for mutants obtained by UV treatment and Figure No.9formutants obtained by MMS treatment.

As many be noted from the Figure No. 8, 9mutants were observed to accumulate >40% of their DCW as lipid. Mutant *B. t*407/UV/5 produced high biomass upon liquid culture going up to 20 g/L of DCW. This mutant also had produced highest amount of PUFA in the first trial-61.96%.Linoleiadic acid (Table 1 and GC chromatogram Figure 6.II). Mutant No. *B.* sps. FPZSP13/UV/5 and Mutant No. *H.* sps.QY113/UV/7 also seem to be promising due to their high biomass, high lipid accumulating capacity and high PUFA producing capability. Comparison of these mutants with results for parental cultures (Figure No.8) dramatically demonstrate the beneficial effect of carrying out UV mutations for improving the PUFA production.



Percentages were calculated on the basis of GC analysis (considering peak area %)

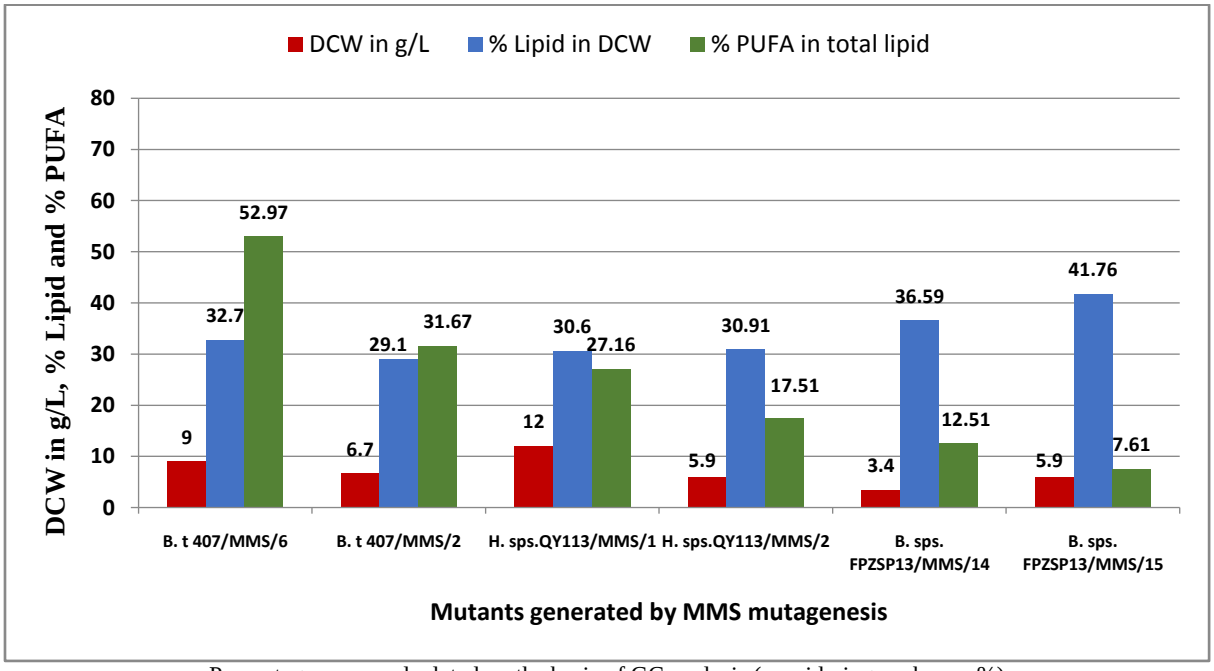
Figure 7. DCW, Percentage lipid in DCW and percentage PUFA produced by parent cultures (20°C, 100 rpm, 120hr).



Percentages were calculated on the basis of GC analysis (considering peak area %)

Figure 8. DCW, Percentage lipid in DCW and percentage PUFA in superior mutants generated by UV mutagenesis (20°C, 100 rpm, 120hr).

The % lipid in DCW and % PUFA for superior mutants generated by MMS Mutagenesis are shown in Figure No. 9.



Percentages were calculated on the basis of GC analysis (considering peak area %)

Figure 9. Percentage lipid in DCW, DCW and percentage PUFA in superior mutants generated by MMS mutagenesis (20°C, 100 rpm, 120hr).

Mutant *B. t* 407/MMS/6 and *H.* sps.QY113/MMS/1 produced high biomass in shake flask fermentation (Figure No.9). Therefore, mutant *H.* sps.QY113/MMS/1, *B. t*407/MMS/2 and *B. t*407/MMS/6 may prove to be potential candidates for industrial application due to their ability to produce high biomass, high lipid accumulating capacity and high PUFA producing capability. Mutations, both chemical and physical, have been used to improve industrial strains for a variety of metabolites. For instance, an increase in the PHB yield of *B.megaterium*Y6, *B. subtilis* K8, and *B. firmus*G2 via mutation has been reported earlier [8].Developing a commercially viable fermentation production process for PUFA using bacteria remains a major challenge though some progress has been made with fungal and algal sources [5,8,16,26].

When our results obtained by use of UV irradiation and MMS mutagenesis are compared with published literature , it is noticeable that similar results displaying superior growth and lipid accumulation in mutants generated by random mutagenesis of *Lipomyces starkeyi* DSM 70296 by UV irradiation were observed by Tapia et al. [9]. Thus, random mutagenesis coupled to a strategic screening program can be considered feasible for the genetic improvement of wild type lipid producing strains [5,15,27].The high commercial values of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids have driven strain-improvement programs using UV-light as mutagenic agent, aimed at increasing the content of those fatty acids in the microalga like *Pavlova lutheri* (SMBA 60) as parent strain [6].Encouraging results obtained by these scientists suggest that random mutagenesis can successfully be applied to increase the yield of n-3 fatty acids by microalgae. Our work is in agreement with such findings as the results of UV Mutagenesis of 3 select parent cultures provided mutants with 3-4 fold increase in PUFA production.

Our results obtained with MMS as a potent mutagen are even more exciting and demonstrate its application in obtaining superior mutants. Literature also supports our suggestion that MMS is very useful mutagen. *Nannochloropsis* sps. a promising marine microalga for biodiesel feedstock production, was treated with the mutagen ethylmethanesulfonate (EMS) in a study by Doan and Obbardto induce mutations to isolate mutants with an enhanced intracellular lipid content [18]. The treatment resulted in up to a four-fold increase in total fatty acid content in the mutant strain [18]. Also, work by Dubey and Vakil on *Bacillus coagulans* using MMS as a chemical mutagen

successfully led to the isolation of phage resistant mutants [21]. The present study of two *Bacillus* sps.(*Bacillus* sps. FPZSP13 and *Bacillus thuringiensis* Bt407) which resulted in improved PUFA production gives support to the idea that MMS is an efficient mutagen for *Bacillus* sps. Moreover MMS proved to be useful for generating improved mutants of a *Halomonas* sps.(*Halomonas* sps. QY113).

Other researchers have reported improved PUFA production in certain bacteria, fungi and microalgal mutant strains [9,11,18,28]. Both PHB and PUFA being related long chain biomolecules produced in nutrient scarcity. It is interesting to explore the possibility of isolating mutants capable of producing a single type PUFA or a combination of valuable PUFA in high concentrations that may allow easier separation processes.

IV. CONCLUSION

In conclusion, classical mutation and selection programmes have been used in the industries for many years and have resulted in large productivity increments which in turn have driven down the cost of production of a wide range of fermentation products. Through the present study we have been able to use UV and MMS mutagenesis and obtain variety of mutants like mutant *B.* sps. FPZSP13/UV/1 making a mixture of 42.10% of LA, AA, ALA, Mutant ,*H*. sps.QY113/UV/2 making 27.89% of 4 PUFA -LA, ALA, ETA, EDA, Another mutant, *H*.sps.QY113/MMS/1 making 27.17% LA as single PUFA. Mutant, *B.t*407/UV/1 is another unique mutant capable of producing 61.96% of Linolelaidic Acid- a closely related structural analogue of PUFA, *B.t*407/MMS/1and *B.t*407/MMS/2 are other promising mutants that make good amount of PUFA consisting of 3-5 fatty acids of interest.

During characterization of mutants it was also noted that the random mutations not only resulted in enhancement/alterations in PUFA profiles but also enhancement in growth as was the case with Mutant No.*B.*sps. FPZSP13/MMS/2, *H*. sps.QY113/UV/2, *H*.sps.QY113/MMS/1,*B.t*407/UV/1 and *B.t*407/MMS/2.These strains may prove to be potential candidates for industrial application with further mutagenesis, media and process optimization. Further mutagenesis program coupled with media optimization and scale up studies to develop a fermentation technology can be expected to result in identification of few of these mutants as suitable candidates for the industrial scale production of PUFA

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