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Effect of physical and chemical mutagens on biopolmer producing strains and RAPD analysis of mutated strains

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ABSTRACT

Mutation (Latin mutare means to change) refers to any heritable change in nucleotide sequence of a gene of the organisms irrespective of altered pheno typic expression of characters of the organisms. Strain improvement can be carried out by physical, such as gamma-rays, x-rays and ultraviolet irradiation, and chemical, such as ethyl methane sulphonate(EMS) and nitroao-methyl guanidine (NTG). The best mutagen and conditions for its use are not identical for all organisms and can only be found by trial and error.The efficiency of alkylating mutagen treated bacterial isolates showed effective reactions depending on the duration of exposure. EMS mainly added the ethyl group to numerous positions on bases of DNA. Out of 20 primers screened, primer A9 was selected on the basis of robustness of amplification, reproducibility, scorability of banding patterns and was employed for diversity analysis. The primer yielded both intense as well as faint bands, and were scored for calculating similarity index values, so as to maximize the number of scorable characters. On the basis of the results obtained, all the four cultures namely, P.putida, B.megterium, E.coli and R.eutropha have higher activity on duration of exposure to UV light. When the period of exposure is for longer duration less will be the survival of the specific cells and all the mutant forms which has the ability to survive alone will remain forever. Similar results were found the Random Amplified Polymorphic DNA (RAPD), a PCR based method which could be used to distinguish strains within a species. Data analysis using bioprofile ID software revealed a variable similarity index between the native isolates and their respective mutants. This clearly illustrates that mutation had occurred in U.V treated and EMS treated isolates. Genetic analysis for identifying mutation using the primer A9 revealed a moderate genetic variability proving mutation.

Key words: Mutagen, UV, EMS, RAPD, Bioprofile.

INTRODUCTION

"Mutagenesis" is the source of all genetic variations. Strain improvement is an essential part of process development for microbial products. It can reduce costs by developing strains with increased productivity. Strain improvement can be carried out by such techniques as rational screening and genetic engineering or by the traditional method of mutagenesis and selection on the basis of direct titre measurement. The last method, also referred to as random screening, is still a reliable and cost effective procedure. Consequently, 'it is frequently the method of choice for short-term strain development.

Terrestrial life as known at present has only been possible by developing a range of protective mechanisms against chemical and physical agents, including UV light, that may result in mutagenesis and the killing of cells. Several types of DNA repair mechanisms have been described in prokaryote and eukaryote cells, correcting a high spectrum of lesions introduced in the DNA molecule. These mechanisms are part of the DNA damage response, a complex system that ensures the maintenance of genomic integrity. DNA damage response consists of checkpoint systems and extensive repair mechanisms that deal directly with DNA damage [9].The problems of environmental pollution caused by indiscriminate dumping of plastic waste have assumed global-proportions. These conventional plastic that are synthetically derived from petroleum are not readily biodegradable. It is considered as environmental harmful wastes.

Biodegradable polymers have attracted a lot of attention in the recent years. These biopolymers are large macromolecules composed of single, repeating monomer units. They are of very high molecular weight and their material characteristics vary according to the nature of their monomer composition. Exopolysaccharides are important constituents of the surface of bacterial cells and play a critical role in the interaction of bacteria with the environment. Many bacteria produce complex Exopolysaccharide (EPS) which can remain attached to the cell surface in a capsular form or be released as slime. Exopolysaccharides producing microorganisms occur widely in nature in different types of habitat.[21] Apart from being biodegradable, some of these biopolymers are being used in the packaging industry, chemical industry, agriculture and medicine. Several carcinogens like dioxins would be released from the plastics when burn and dangerous for people who inhale it. When buried, it blocks the supply of air and water to the soil [12].

Plastic enter into the marine habitat and kills millions of marine animals like turtle, fishes etc. Recent studies revealed that about 10,00,00 marine animals die every year from the ingestion of plastic [10]. Molecular structure of PHB does not depend on the features of the strain and condition of carbon nutrition of microorganisms producing PHB. PHB was originally shown to be a constituent of lipid inclusions in the cells of *Bacillus*. It can be extracted with hypochlorite solution, which is highly degradative procedure and decreases the molecular weight of the isolated granules presumably by the removal of their outer layer [25].

Studies with UV-sensitive mutants in many organisms have been a help in the understanding the repair pathways and syndromes associated with deficiencies in the DNA repair mechanisms. Excision repair mutants sensitive to UV light were first studied in prokaryotes, mainly in *Escherichia coli* and, during the last decade, in eukaryotes. In *Saccharomyces cerevisiae* a large

number of radiation sensitive mutants (*rad*) have been identified as being involved in NER [11, 17].

Random Amplified Polymorphic DNA (RAPD) is a PCR based method which can be used to distinguish differences between strains within a species. One or a few short primers of arbitrary sequence are allowed to bind under low stringency conditions to various sites on both strands of the template DNA. The PCR reaction yields a series of products of varying size, which may be separated by gel electrophoresis. The band pattern represents a "genetic fingerprint" characterizing a particular bacterial strain [26].

The present investigation was made to in biotechnological aspects, cheap substrates, mutations and genetically modified high PHB yielding bacteria or plants can be used in biopolymer production technique to improve the strains by using both physical and chemical mutagen and after treatment they were analyzed by RAPD technique to confer the potential changes occurred on their chromosomal element similarly observed the quality and quantity of PHB.

MATERIALS AND METHODS

UV mutagenesis (Miller, 1992)

Procedure

Overnight broth culture (*P.putida, E. coli, B. megaterium* and *R.eutropha*) was centrifuged at 10,000 rpm for 15mins at room temperature. Supernatant was discarded and the residual pellet was resuspended in 1ml sterile saline. The bacterial suspension was then serially diluted up to 10^{-9} from which dilution at 10^{-6}, 10^{-7} and 10^{-8} were spread on air dried LB agar plate for total viable count (TVC).

Further a quantity of 5ml of the same was transformed to a sterilized petri plate exposed to U.V under sub lethal time (3 min, 5min and 7 min). On completion the plate was covered with lid and black cloth for the purpose of incubation in darkness for a period of 2 to 4 hrs to ensure that the implantation could be serially diluted of mutant in the subsequent generation. Out of this a quantity of 1ml was taken and serially diluted and 0.1 ml each of 10^{-1}, 10^{-2} and10^{-3} of the dilute was spread on LB agar and containing was taken 3 min interval. The same exercise was repeated for 5 min and 7 min and all the results were tabulated.

EMS (Ethyl Methyl Sulphonate) Mutation (Miller, 1992)

Procedure

The overnight culture was diluted at the ratio of 1:20 in LB broth. Out of which 5 ml of the suspension taken and incubated at 37ºC for two hours. Then it was washed with tris buffer and resuspended with 2.5ml of tris. Mixture was divided into five parts of 0.5 ml each. Each one was added with 30µl of EMS in different time intervals. Out of them the result of three intervals were taken for recorded (30 min, 45 min and 1hr).

Total Viable Count for U.V and EMS

Procedure

5 ml of the overnight cultures were diluted to 1:20 ratio maintained for 2 hrs at 37^{o} C. After 2 hrs 5ml was taken out and washed twice with tris buffer and again resuspended in 2.5ml of tris.

From this 1ml was taken and serial diluted from 10^{-1} to $10^{-8.}$ Plating was done on Luria Bertanii for total viable count.

Calculations

1. Frequency of induced mutants =

$$\frac{\text{Total number of colonies (U.V. exposed in different time intervals)}}{\text{Total viable count}}$$

2. Percentage of induced mutants=

$$\frac{\text{Total number of colonies (U.V .exposed in different time intervals)}}{\text{Total viable count}} \times 100$$

3. Frequency of induced mutants=

$$\frac{\text{Total number of colonies (EMS exposed in different time intervals)}}{\text{Total viable count}}$$

4. Percentage of induced mutants=

$$\frac{\text{Total number of colonies (EMS exposed in different time intervals)}}{\text{Total viable count}} \times 100$$

RAPD Analysis of Physical and Chemical Mutagen Treated Bacterial Strains

DNA Isolation (Phenol Chloroform method)

15ml of nutrient broth culture of different isolates *P.putida, E. coli, B. megaterium* and *R.eutropha* were centrifuged at 10,000 rpm for 10 min. The resultant pellet was resuspended in15ml of sterile distilled water and centrifuged at 10,000 rpm for 10min. Then it was then collected and ground with 300µl of CTAB (cetyltrimenthyl ammonium bromide) DNA extraction buffer (1% W/V CTAB;1.4M NaCI; 10mM EDTA, pH 8.0; 100mM Tris-HCI, pH 8.0; 0.2% V/V β-mercaptoethanol) in a glass homogenizer The mixture was emulsified with equal volume of phenol:choloroform (1:1). It was centrifuged at 10,000 rpm for 5 mins at room temperature. The aqueous phase was collected and mixed with equal volume of chloroform, isoamyl alcohol (24:1). The mixture was then centrifuged at 10,000 rpm for 5min at room temperature. The aqueous was collected, washed and added with equal volume of cold absolute ethanol and the DNA was allowed to precipitate by keeping the tubes in -20ºC for overnight. DNA pellets were obtained by centrifugation at 10,000 rpm for 5min and the ethanol was air-dried. The pellet was dissolved in 50µl of TE buffer (Tris 10mM, pH 8.0 and EDTA 1mM, pH 8.0). The quality of the isolated genomic DNA was tested by agarose gel electrophoresis.

RAPD-PCR Analysis

20µg of genomic DNA was dissolved in 20µl PCR reaction buffer containing 10mM Tris-HCI(pH 9.0), 1.5mM $MgCI_2$, 50mM KCI, 0.01% gelatin, 0.2mM dNTPs, 21 pM of primer and 0.5 U of DNA polymerase. Twenty primers (RAPD Kit A1 to RAPD Kit A20) obtained from IDT were used for RAPD-PCR studies. PCR was conducted according to the methods of initial heat step (94ºC for 5min.), 40 cycles of denaturation (94ºC for 1min), annealing (36ºC for 1min) and extension (72ºC for 2min.) and a final Extension step (72ºC for 7min). Amplification was performed using a programmable thermal cycler PTC-150 (MJ Research, USA). The products of PCR and DNA size markers (λ DNA digested with EcoRI and HindIII (Bangalore, Genei) were loaded onto a 1.6% tris-broate-EDTA [19] agarose gel and run for 4 hrs. At 50V, the gels were stained with ethidium bromide and photographed. Each lane of RAPD profiles was subjected to gel documentation system (Vilbret-lourmat, France).

Table 1. Sequence of the Primers used for RAPD

Primer code	Sequence
RAPD Kit A1	5' CAGGCCCTTC 3'
RAPD Kit A2	5' TGCCGAGCTG 3'
RAPD Kit A3	5' AGTCAGCCAC 3'
RAPD Kit A4	5' AATCGGGCTG 3'
RAPD Kit A5	5' AGGGGTCTTG 3'
RAPD Kit A6	5' GGTCCCTGAC 3'
RAPD Kit A7	5' GAAACGGGTG 3'
RAPD Kit A8	5' GTGACGTAGG 3'
RAPD Kit A9	5' GGGTAACGC 3'
RAPD Kit A10	5' GTGATCGCAG 3'
RAPD Kit A11	5' CAATCGCCGT 3'
RAPD Kit A12	5' TCGGCGATAG 3'
RAPD Kit A13	5' CAGCACCCAC 3'
RAPD Kit A14	5' TCTGTGCTGG 3'
RAPD Kit A15	5' TTCCGAACCC 3'
RAPD Kit A16	5' AGCCAGCGAA 3'
RAPD Kit A17	5' GACCGCTTGT 3'
RAPD Kit A18	5' AGGTGACCGT 3'
RAPD Kit A19	5' CAAACGTCGG 3'
RAPD Kit A20	5' GTTGCGATCC 3'

Data Analysis

The RAPD data was analyzed using Bioprofile ID software. A genetic similarity (GS) was computed based on Jaccard's coefficient of similarity. Each RAPD fragment was treated as a unique character and was scored as 1 (present) or 0 (absent). The 1/0 matrix was prepared for all fragments and used to generate Jaccard's coefficient of similarity and recorded accordingly.

RESULTS

Table. 2 Total viable count of desired bacterial isolates

Dilutions	Total Viable Count (TVC) cfu/ml			
	P.putida	***B.megaterium***	***E.coli***	***R.eutropha***
10^{-6}	226	201	218	200
10^{-7}	162	103	143	107
10^{-8}	62	79	55	47

Table. 3 U.V. mutagenesis of *P.putida* at different time intervals

Dilutions	Duration to UV exposure (minutes)		
	3'	5'	7'
10^{-1}	55	34	-
10^{-2}	13	2	-
10^{-3}	-	-	-

Table. 4 .E.M.S.treated *P.putida* at different time exposure

Dilutions	Duration to EMS exposure (minutes)			
	15’	30’	45’	60’
10^{-1}	42	7	-	-
10^{-2}	-	-	-	-
10^{-3}	-	-	-	-

Table.5. U.V. mutagenesis for *B. megaterium* at different time intervals

Dilutions	Duration to UV exposure (minutes)		
	3’	5’	7’
10^{-1}	50	30	-
10^{-2}	10	1	-
10^{-3}	-	-	-

Table.6. Treatment of E.M.S. on *B. megaterium* *at* different time exposure

Dilutions	Duration to EMS exposure (minutes)			
	15’	30’	45’	60’
10^{-1}	30	5	-	-
10^{-2}	-	-	-	-
10^{-3}	-	-	-	-

Table.7. U.V. exposure to *E.coli* at different time intervals

Dilutions	Duration to UV exposure (minutes)		
	3’	5’	7’
10^{-1}	75	43	-
10^{-2}	14	4	-
10^{-3}	-	-	-

Table.8. E.M.S. treatment on *E.coli* at different time exposure

Dilutions	EMS treatment(minutes)			
	15’	30’	45’	60’
10^{-1}	53	2	—	—
10^{-2}	—	—	—	—
10^{-3}	—	—	—	—

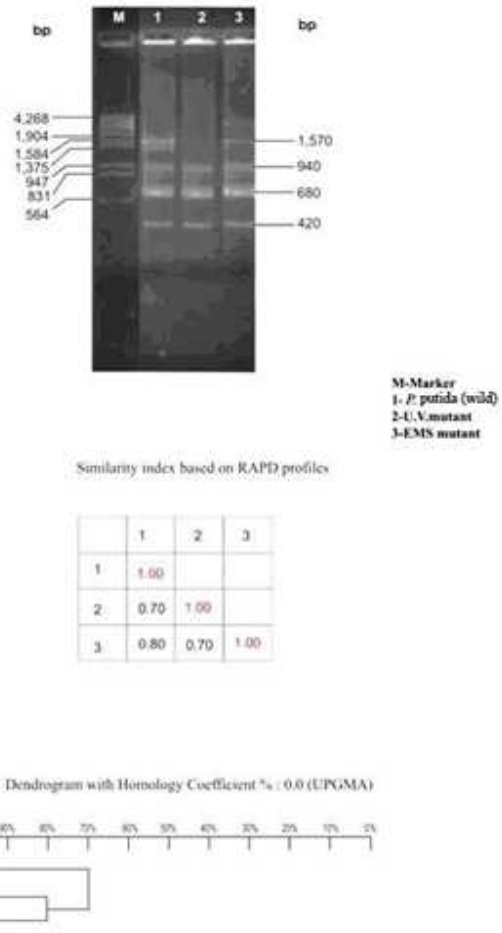
Table.9. U.V. exposure to *R.eutrsopha* at different time intervals

Dilutions	Duration to UV exposure (minutes)		
	3’	5’	7’
10^{-1}	47	33	-
10^{-2}	16	4	-
10^{-3}	-	-	-

Table.10. EMS treatment on *R.eutropha* at different time exposure

Dilutions	Duration to EMS exposure (minutes)			
	15'	30'	45'	60'
10^{-1}	33	15	-	-
10^{-2}	-	-	-	-
10^{-3}	-	-	-	-

Figure: 1 Random Amplified Polymorphic DNAs of Isolated Fragments (*P. putida)* by using Different RAPD Primer Kit9

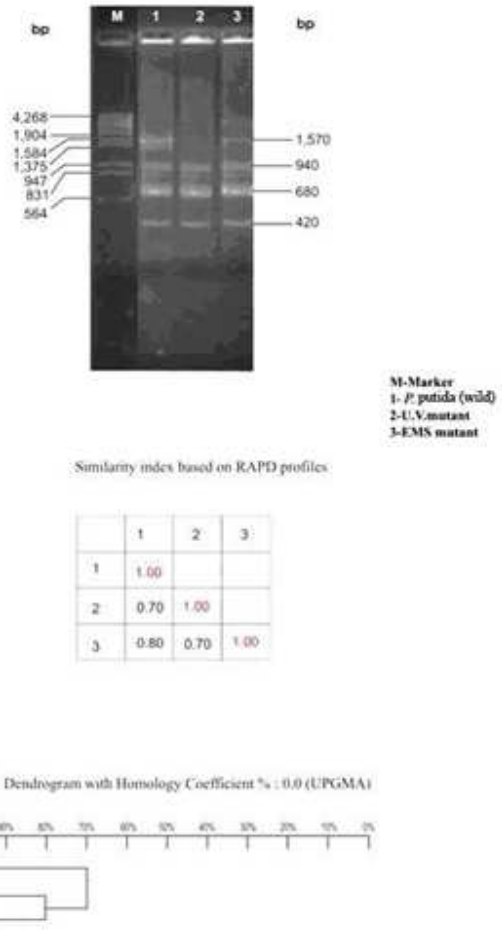


As per the calculations the frequency of *P.putida* (exposed under U.V at different time intervals) was 34×10^{-1} cfu/ml and the percentage was 3.4% (Table 3). Similarily the organisms were treated with EMS at different time intervals and the result was 8.09×10^{-3} cfu/ml and the percentage was recorded as 0.80 % (Table 4). The total viability count of the isolates are depicted in Table 2.

In the case of *B.megaterium* under the same method the frequency was 4.30×10^{-10} cfu/ml and the percentage was 4.30. It was treated with EMS at different intervals yielding a result of 3.79×10^{-10} cfu/ml and the percentage was found to be 3.79 % (Table 5 & 6).

E. coli was exposed under different time intervals and the results were recorded. The frequency was 2.55 $\times 10^{-11}$ cfu/ml and the percentage was 2.54 %. Under the treatment of EMS the result was 3.63 $\times 10^{-11}$ cfu/ml and percentage was 3.63 % (Table 7 & 8).

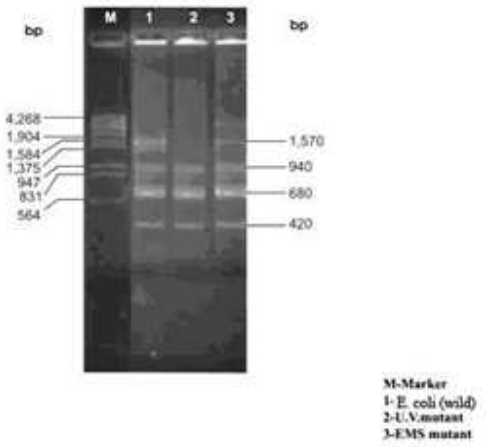
Figure: 2 Random Amplified Polymorphic DNAs Of Isolated Fragments (*B. megaterium)* By Using Different RAPD Primer Kit9



The mutation frequency was calculated for the *R.eutropha* for both UV and EMS at different time intervals. It was observed that in the UV the frequency was 7.02 $\times 10^{-10}$ cfu/ml and the percentage was 7.02%. In the same time intervals EMS mutant frequency was 3.19$\times 10^{-10}$ cfu/ml and 3.19% was observed (Table 9 & 10).

In the present study the RAPD Analysis was performed for the four physically and chemically treated isolates with RAPD kitA9 for their ability to differentiate intra species variation. The primer RAPD kit A9 amplified *P.putida*, with two fragments of 831 base pairs (bp) and another fragment with the magnified size of 354.

Figure: 3 Random Amplified Polymorphic DNAs Of Isolated Fragments (*E.coli)* By Using Different RAPD Primer Kit9



Similarity index based on RAPD profiles

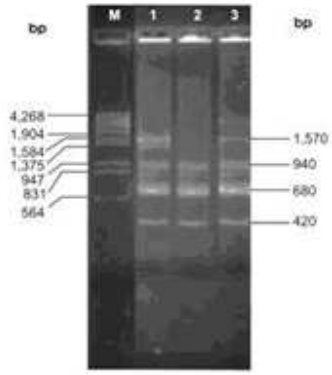
	1	2	3
1	1.00		
2	0.70	1.00	
3	0.80	0.70	1.00

In UV treated strains the RAPD fragment recorded high amplified low intensity base pairs of 564 and 344. In chemical treated (EMS) strains the comparison was of very higher range of 1672 to 225 base pair with four fragment range. The visualization of band structure was 1670 in the first band and in second and the third was 1210 was highly exploited with 810 base pairs (bp) and in the four was lowered with 345 base pairs with high intensity.

The RAPD profile of wild *P.putida* was seen as 1.00% and in U.V and it was low as 0.30% and in EMS was 0.80%. (Fig: 1).

Dendrogram in the phylogram form deficiting the phylogenetic relatedness between various isolates and Nel's holomology genetic coefficient generated by the Unweighted Pair Group method with Arithmetic Mean (UPGMA) using arithmetic average.. The study showed that wild *P. putida* and U.V and EMS mutant are clustered together as same species of 20% U.V and 70% EMS (Figure 1).

Figure: 4 Random Amplified Polymorphic DNAs Of Isolated Fragments (*R. eutropha)* By Using Different RAPD Primer Kit9



M-Marker
1-R. eutropha
2-U.V.mutant
3-EMS mutant

Similarity index based on RAPD profiles

	1	2	3
1	1.00		
2	0.70	1.00	
3	0.80	0.70	1.00

In case of *B megaterium* the random amplified polymorphic DNA's of the strains were treated with UV and EMS. The native strain visualized 3 clear fragments and one incomplete or partial fragment size with the length of 1,904 while in the marker DNA. The other 3 fragment, one at the top (hevier one) recorded molecular weight of about 1,584 and 2nd one was visualized just above the base pair (bp) 564 and the last one fall on the same molecular size of 410s.

U.V.treated also yielded four different fragments among which three were clearly visualized and one was partially visualized. Heavier fragment had the similar size of base pair length when compared with native strain in between the range of 1904 and 1,584 the second one was uncleared fragment placed on the side of the fragment which was in between the 831 and 564.The third fragment of the U.V. treated *B. megaterium* visualized on the region of just above the 564 and the fourth one was present with the similar length of 564 and 410.

The similarity index based on RAPD profiles of native and UV, EMS treated *B. megaterium* shows the same variation of 65% on both the treated strain of *B. megaterium* (Figure 2). The maximum similarity index was recorded inbetween the strains of native and U.V. treated was 80%.

The dendrogram with homology % of UPGMA of native and mutagen treated strains of *B. megaterium* had shown 80% similarity. With wild of *B.megaterium* RAPD analysis with kit 7 without treatment yielded 5 different molecular weight fragments. Intrestingly the U.V treated strains showed only three fragments which confirmed that there must have been deletion mutation. Simultaneously the EMS treated *B.megaterium* shows 5 different fragments which were not on the same molecular size fragments of the wild type strain. Here also the confirmations of the chance for the mutation occur on their genetic element.

Random amplified polymorphic DNA's of *E.coli* wild and U.V.,EMS treated isolates generated with primer kit RAPD kit A9 showed up with 4 fragments of below 1,850 base pairs, 800 (bp), 560 bp and 380 base pairs (bp) respectively (Figure 3)

Intrestingly the U.V treated *E.coli* strains yielded two fragments with minimum variation of fragment size in between the range of 1,850 and 1,570. The other two fragments had shown the similar molecular size fragmentation of 560 and 380. The third fragment of *E.coli* (treated with EMS) also recorded the same fragment visualization of U.V. treated *E.coli* strain with four different fragment size. Both the U.V and EMS treated *E.coli* strain similarity index based on RAPD profiles had 0.75 with U.V. treated and 0.60 of EMS treated respectively.

The U.V treated *E.coli* strain has higher similarity index of 0.5 with wild strain. No such significant variation was observed in between the EMS treated strain and wild. UPGMA dendrogram with Homology coefficient % of all three (wild, U.V and EMS treated) *E.coli* (Fig: 3) indicate the % of fragment variation occurred in between the strains of wild and EMS treated *E.coli*. The physical mutagen of U.V does not yield a significant alteration of the strain or the strain could overcome the extreme treatment of the agent.

Similarity index based on RAPD profile for the *R.eutophus* shows 0.70% in U.V treated and 0.80 in EMS treated strains. With the reference to the marker DNA the mutation occurs in between the base pair (bp) range of 4,268 to 947 (deletion mutation) (Figure 4).

In case of EMS treated strain of *R.eutophus*, first fragment was of in the region between 4,268 bp and 1,904 bp. But the second band contained 1,570 (bp) third 940 (bp), fourth 680 (bp) and final one was 420 (bp). The probable chance for the mutation site was located just above the second fragment of 1,570 bp. The first band did not show any dissimilarity between the native and U.V treated strains. Remaining 3^{rd}, 4^{th} and 5^{th} band resembled the U.V treated and native strains. Dendrogram with homology coefficient % of UPGMA for the *R.eutophus* reflects there was a chance for the occurrence of mutation in U.V treated and EMS treated *R.eutophus* for 30% and 20% respectively.

The study included the analysis of four bacterial isolates that were subjected to strain improvement. RAPD analysis was performed to determine the similarity between wild strain, UV treated strain and EMS treated strain.

DISCUSSION

The use of PHB as a substitute for non biodegradable petroleum-based plastics is substantially more expensive than using fossil-based counterparts and offers no performance advantage other than biodegradability. Furthermore, the continuous depletion of petroleum sources has emphasized the need for biodegradable microbial plastics. Today, most research efforts in this field concentrate on the isolation of PHB-producing microorganisms from different sources and improvement in the PHB production ability of microorganisms via less expensive substrates and genetic modification for high-yield biopolymer production.

Mutations, both chemical and physical, have been used to improve industrial strains. UV light has been shown to be lethal and mutagenic in a variety of organisms, including bacteria [2, 4, 7, 23]. [8] reported an increase in the PHB yield of *B. megaterium* Y6, *B. subtilis* K8, and *B. firmus* G2 via mutation. [3] also studied D(-)-3-hydroxyalkanoate in 11 different *Bacillus* spp.and observed that PHB accounted for 50% of DCW in the bacteria UV light has been shown to be lethal and mutagenic in a variety of organisms including a wide range of bacteria. [1] concentrated on increasing PHB yields to this level in other bacteria using mutations. This study yielded a putative UV mutant strain (B8) of *Bacillus thuringiensis* IAM 12077, with increased cell density (3.5-fold), a concomitant increase in PHB production (from 0.24 g/l to 1.3 g/l), and a 1.5-fold increase in accumulation (from 16% to 24.5%), as compared to the parental strain. The cyclobutane pyrimidine dimers and pyrimidine or photoproducts are the most important premutational DNA lesion induced by U V radiation [5, 15]. Other lesions such as DNA strand breaks and thymine glycols are also induced by UV treatment [6, 13]. The correlation between the quantity of energy absurbed by DNA and the observed biological effect is illustrated with in the 240 nm wavelength region. On the basis of the results obtained, all the four cultures namely, *P.putida, B.megterium, E.coli* and R.eutropha have higher activity on duration of exposure to UV light. Slower and shorter duration of exposure have the least action on thymine-thymine dimer bond formation. When the period of exposure is for longer duration less will be the survival of the specific cells and all the mutant forms which has the ability to survive alone will remain forever, the percentage observed in the findings for *P.putida* (20% UV, 70% EMS) in Table 4.3 and 4.4, for *B.megterium* (65% UV, 85% EMS) in Table 4.5 and 4.6, for *E.coli* (75% UV, 85% EMS) in Table 4.7 and 4.8 and *R.eutropha* (70% UV, 80% EMS) in Table: 4.9 and 4.10. At a given point in time, several clones, or strains, of the same species may also coexist. For example, an individual typically harbours one to ten different *E. coli* strains simultaneously [16, 23]. Some of these strains have the capacity to persist in the colonic microflora for extended periods of time (resident strains), while others are not capable of long term colonization (transient strains) [20]. *E. coli* strains in the normal intestinal microflora have been identified by extended serotyping, by Multi Locus Enzyme Electrophoresis (MLEE) [25] or by biotyping [18, 21].

However the absorption at 240 nm showed efficiency as the mutants were highly indirect. Cellular cytochromes belonging to the respiratory system had shown maximum absorptions in this spectral range constituting the primal photoreceptors.

The banding profile of *P.putida* (Fig 1) and its mutants yielded a total of 10 amplication products, out of which 2 bands of size 810 & 345bp were found in all the strains. The band of size 1670 bp was found only in mutants. Two bands of size 765bp and 225bp were only found in

the UV mutant. Bands of size 1670bp and 1210bp were found only in EMS mutant. Data analysis of DNA polymorphisms on *P.putida* and its mutants showed a very low similarity index of 0.30 between the isolate and U.V mutant. A high similarity index of 0.80 was observed between the isolate and EMS mutant (Fig: 1).

The banding profile of *Bacillus megaterium* and its mutants yielded a total of 9 amplication products, out of which 2 bands of size 1580 & 410bp were found in all the strains. The band of size 650bp was found only in the native strain (Fig: 2).

The banding profile of *E.coli* (Fig 3) and its mutants yielded a total of 12 amplication products, out of which 2 bands of size 560 & 380bp were found in all the strains. The bands of size 1850bp & 1570bp were found only in mutants. Two bands of size 800bp and ~1700bp were found in the native isolate. Data analysis of DNA polymorphisms on *Bacillus megaterium* and its mutants showed a similarity index up to 0.80 (0.65 between the isolate and both mutants of UV and EMS treatment and 0.80 between the mutants) proving mutation.

The banding profile of *R.eutropha* and its mutants yielded a total of 12 amplication products, out of which 3 bands (420, 680 & 940bp) were found in all the strains. The band of size 1570bp was found in the native and EMS treated mutant. A band of size ~1800bp was only found in the EMS mutant. Data analysis of DNA polymorphisms on *R.eutropha* and its mutants showed a similarity index up to 0.80 (0.70 and 0.80 between the isolate and the mutants of U.V and treatment 0.70 between the mutants) respectively proving mutation (Fig 4). Data analysis using bioprofile ID software revealed a variable similarity index between the native isolates and their respective mutants. This clearly illustrates that mutation had occurred in U.V treated and EMS treated isolates. Genetic analysis for identifying mutation using the primer A9 revealed a moderate genetic variability proving mutation.

CONCLUSION

The efficiency of alkylating mutagen treated bacterial isolates showed effective reactions depending on the duration of exposure. EMS mainly added the ethyl group to numerous positions on bases of DNA. The additions to the O^6 position of guanine and the O^4 position of thymine were the most potentially mutagenic since they cause direct mispairing specificity of EMS in that it caused G: C A: 9 transitions almost exclusively among base substitutions in the case of *P.putida* (Table: 4), *B megaterium* (Table: 6), *E.coli* (Table: 8), and *R.eutopha* (Table: 10) pinpointing the 0^6 alkyl guanine as the most relevant biological premutational lesion. The frequency of *P.putida* (exposed under U.V at different time intervals) was 34×10^{-1} cfu/ml and the percentage was 3.48%. Similarly the organisms were treated with EMS at different time intervals and the result was 8.09×10^{-3} cfu/ml and the percentage was recorded as 0.80 % (Fig.1).In the case of *B.megaterium* under the same method the frequency was 4.30×10^{-10} cfu/ml and the percentage was 4.30. It was treated with EMS at different intervals yielding a result of 3.79×10^{-10} cfu/ml and the percentage was found to be 3.79 % (Fig.2).*E. coli* was exposed under different time intervals and the results were recorded. The frequency was 2.55×10^{-11} cfu/ml and the percentage was 2.55 %. Under the treatment of EMS the result was 3.63×10^{-11} cfu/ml and percentage was 3.63 % (Fig.3).The mutation frequency was calculated for the *R.eutropha* for both UV and EMS at different time intervals. It was observed that in the UV the frequency was

7.02 $\times 10^{-10}$ cfu/ml and the percentage was 7.02%. In the same time intervals EMS mutant frequency was 3.19×10^{-10} cfu/ml and 3.19% was observed (Fig.4). UV treated strains showed much stability than those exposed to EMS. Hence it can be concluded that UV treated strains are much preferred for PHB production and further studies. RAPD analysis showed homology between wild and mutated strains.

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