COMPARATIVE ANALYSIS BETWEEN WILD TYPE AND MUTATED *BACILLUS MEGATERIUM* STRAIN WITH RESPECT TO PHB PRODUCING GENES

Monika Sharma[1*] and Harish Kumar Dhingra[1]

[1]Department of Science, CASH, Mody University of Science and Technology, Lakshmangarh 332311, Rajasthan, India

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ABSTRACT

Modern life style contributes to higher use of plastics. The higher use of synthetic plastics increases the non-degradable garbage which can't be degraded by any chemical or microbial degradation process. Poly-β-hydroxybutyrate is naturally occurring biopolymer produced by verity bacteria. It has gained major importance due to its structural diversity and close analogy to synthetic plastics. In the present study, non-discriminate isolation of bacteria from various environmental sources was carried out. Out of them highest PHB producing strain was subjected to characterization of PHB producing genes *phaB* and *phaC*. Mutagenesis was used for increase the PHB production. The genes sequences of *phaB* and *phaC* from wild type and mutant type were compared. An effort to understand and analyze the genes involved in production of PHB is the main effort of this study. This research unfolds 'New Hope' in the field of biodegradable alternatives to plastics.

Key words: Biodegradable, Bioplastic, Polymer, Inclusion body, Poly-β-hydroxybutyrate

No: of Tables: 9 **No: of Figures:10** **No: of References:39**

INTRODUCTION

Polymers are the macromolecules that are made of small molecules arranged in repeating structure. Polymers are natural and synthesized compounds. Plastic is a petroleum based synthetic, semi synthetic or natural polymer that is used to make filaments, coatings and adhesives. Now a days modern life style contributes to higher use of plastics. A number of packed products like bottled water, milk, juices, coke and instant vegetables, etc. make use of synthetic plastic in packaging process. The higher use of synthetic plastics increases the non-degradable garbage which can't be degraded by any chemical or microbial degradation process. The higher molecular weight of these plastics and lacks of depolymerase enzyme make their resistant to degradation process. The management of these synthetic plastics increases the burden on municipal department of the country. Municipal departments either dump these wastes in the soil or incinerate which will make these dumping lands barren and cause the gaseous pollution in environment. Various hazardous chemicals like hydrogen cyanide generated during incineration process, cause dangerous human health issues and environmental pollution. The accumulation of plastic carry bags in a low lying area might have adverse effect on health of grazing animals, if swollen or eaten. The Recycling of these plastics is a very tedious and costly process. Only 1-2% of plastic bags end up getting recycled. It is expected that thousands of marine animals and more than one million birds die each year as result of plastic bags (Heap 2009). Often, pieces of plastic litter ingested by animals, clogging their intestines which results in the death by starvation. Birds become entangled in plastic bags and can't fly as a result finally die. Biodegradable plastic was discovered as an alternative of synthetic plastic. Biodegradable plastic is often known as natural polymer and it is formed from the polymerization reactions of activated monomers from various complex metabolic processes. Biodegradable plastics are either partly or fully degraded either by non-enzymatic hydrolysis or by microbial enzymes conducted process (Poirier et al. 1995). Different types of biodegradable plastics include polylactic acids (PLA), polyhydroxyalkanoates (PHA) and poly propylene (Patwardhan and Shrivastava 2004; Datta et al. 1995). Among these type PHA is most promising biodegradable plastic which is fully biodegradable and do not have any defects faced by other types. PHB is most common type of PHA and the ability of bacteria to accumulate PHB is often used as a taxonomic characteristic (Kim et al. 1992). The PHB is non-toxic, biocompatible and biodegradable thermoplastics that can be produced from renewable resources (Reddy el al. 20003). They have a high degree of polymerization; are highly crystalline, optically active and insoluble in water. These features make them highly competitive with polypropylene, the petro chemically derived plastics (Lee et al. 2000; Park el al. 2002). PHB is a kind of polyesters synthesized by various microorganisms as energy storage materials under adverse conditions, i.e.

limitation of some essential nutrients or excess availability of carbon source (Lee 1996). PHB, synthesized by many Gram positive and Gram negative bacteria such as *Rhizobium* sp., *Bacillus megaterium*, *Azotobacter* sp. and *Pseudomonas* sp. storage compounds, are deposited as insoluble inclusions in the cytoplasm (Steinbuchel and Valentin 1995). During nitrogen starved conditions, reduced amino acid synthesis accompanied by increased acetyl CoA and increased activity of β-ketothiolase, which promote PHB accumulation. In such starved condition, concentration of acetyl phosphate increases and finally PHB synthase enzyme is activated (Asada et al. 1999). McCool and Cannon reported PHA synthase of *Bacillus megateruium* as a unique one and are considered as the newer type (McCool and Cannon 1999). Mutation in genes of interest plays important role increasing in PHB production. Improvement of strains by mutation is very cost effective method and strategically better approach for getting maximum output of PHB. As mineral oil prices continue to rise the world is being forced to use biodegradable PHB in many applications. Efforts have been made to use them in agricultural, medical, packaging industries.

The current piece of work was undertaken for development of high PHB producing mutant bacterial strain and comparative analysis of PHB synthesis gene sequences of wild type and mutant bacterial strain.

Materials and Methods

Sampling, screening and selection of highest PHB producing bacterial isolate

To isolate potential PHB producing isolates, samples were collected as green gram's root nodules, rhizospheric soil of the grassy garden, compost from the municipal composting site, waste sample from municipal waste disposal area, mud soil from collected water in soil, salt water sample from salt making site, detergent mixed domestic water sample and sewage water from municipal liquid waste disposal site. The representatives of pure bacterial isolates were screened for the presence of PHB granules intracellularly using carbol fucshin and lipophilic Sudan Black B Stain. Quantification of PHB production by selected isolates was done and maximum PHB producing isolate was selected. The complete methodology regarding these experiments can be found out in our previous publication (Sharma and Dhingra 2015).

Development of mutant strains for increasing PHB production

The physical mutagen (UV) and chemical mutagen (EMS) were used for making mutant of *Bacillus megaterium* (M.com2(1)) for increase in PHB production.UV mutagenesis- The *Bacillus megaterium* (M.com2(1)) culture was prepared by transferring a loopful of inoculum to the nutrient broth and keeping it for 24h at 30°C under shaking conditions (100rpm). From this, 1.0ml inoculum was serially diluted upto 10^{-4} and spread on nutrient agar plates. The plates were exposed to UV radiations for different time (30sec to 15min) at distance of 10cm from UV irradiation

source of 235nm. After exposure to UV irradiations, the petri plates were immediately wrapped in a black paper to avoid recovery of mutation. The colonies were sub-cultured and screened for PHB production by two methods, one by Sudan Black B plate method and the other by quantification of PHB produced as described in previous sections (Patnaik et al. 2002).

Chemical mutagenesis- For mutation studies, 60µl of EMS was transferred to individual sterilized centrifuge tubes containing 1.0ml of overnight grown bacterial culture. These tubes were incubated at 30°C for 5-60min. After this, the tubes were centrifuged at 8000rpm for 15min. The supernatant was discarded to remove the EMS. The pellet was washed with saline water to remove the traces of EMS from bacterial cells. The pellet was suspended in 1ml of phosphate buffer (pH 7.0). The suspension was serially diluted upto 10^{-4} dilutions. An aliquot of 100µl from 10^{-4} was spread on the petri plates containing nutrient agar. Plates were incubated at 30°C for 24-48h. The petri plates with isolated colonies were selected for further studies (Sreeju et al. 2011). These colonies were sub-cultured in nutrient broth. After that colonies were screened for PHB production by two methods, one by Sudan Black B plate method and the other by quantification of PHB produced as described in the previous sections.

Identification of PHB producing genes

Designing of gene specific primers- A PCR amplification technique was used to amplify the PHB biosynthesis genes from *Bacillus megaterium* (M.com2(1)) (best PHB produced in the study) wild type and its mutant. Various primers were designed and synthesized using sequence data available at gene bank (www.ncbi.nlm.nih.gov) (Berekaa 2012). The list of primers used in the study is given in tab. 1.PCR cloning of *phaB* (Acetyl CoA reductase)- All the PCR reactions were carried out in thermocycler: Gene Amp PCR system from Whatman Biometra. The *phaB* gene, which code for the acetyl CoA reductase, was amplified from the genomic DNA of *Bacillus megaterium* (M.com2(1)) wild type and its mutant by PCR. A total of 15µl reaction mixture was prepared using the forward and reverse primers of *phaB* gene and then subjected to PCR (Tab. 2). Polymerase chain reaction for *phaB* gene- PCR cocktail for *phaB* gene amplification: The composition of PCR reaction mixture for amplification of *phaB* gene sequence in *Bacillus megaterium* (M.com2(1)) wild type and its mutant was summarized in tab. 3. The contained was mixed and the reaction was carried out in thermocycler. PCR cloning of *phaC* (PHA synthase)- The PCR reactions were carried out in thermocycler: Gene Amp PCR system from Whatman Biometra. The *phaC* gene, which code for synthase, was amplified from the genomic DNA of *Bacillus megaterium* (M.com2(1)) wild type and its mutant by PCR. A total of 15µl reaction mixture was prepared using forward and reverse primers of *phaC* and then subjected to PCR (Tab. 4).

Polymerase chain reaction for *phaC* gene- PCR cocktail for *phaC* gene amplification: The composition of PCR reaction mixture for amplification of *phaC* gene sequence in *Bacillus*

megaterium (M.com2(1)) wild type and its mutant was as summarized in tab. 5. The contained was mixed and the reaction was carried out in thermocycler.

Examination of PCR product by agarose gel electrophoresis- A 3µl of PCR product from each PCR reaction vial was analyzed in 1% agarose gel electrophoresis process. The size of the *phaB* and *phaC* gene amplicons were determind by comparing with a 100bp DNA ladder (Banglore Genei, India). The PCR products were stored at -20°C for further sequencing.

Sequencing of amplicons- Sequencing was performed by commercial services through Bionic Corporation, Jaipur. DNA sequencings were carried out using primer set PhaB (forward) and PhaB (Reverse) for *phaB* gene and primer set PhaC (Forward) and PhaC (Reverse) for *phaC* gene by Sanger sequencing method. The reactions were carried out in an automatic DNA sequencer (ABI prism, USA) at Avantor sequencing services, Chennai.

Nucleotide sequence submission- The homology of *phaB* and *phaC* gene sequences were compared with the other *phaB* and *phaC* gene sequences from the gene databases of the National Center for Biotechnology Information (NCBI) through the nucleotide Basic Local Alignment Search Tool (BLAST) server (www.ncbi.nlm.nih.gov/BLAST). These gene sequences were submitted in NCBI through Blankit.

Comparative analysis between wild type and mutant with respect to PHB producing genes

To compare the wild type and mutated *phaB* & *phaC* gene sequences, the online bioinformatics sequences analysis program ClustalW was used (http://www.genome.jp/tools/ClustalW). Both wild type and mutated gene sequences were entered into the ClustalW and comparison alignment was run (Han et al. 2003). The results were examined to identify whether a single nucleotide polymorphism (SNP) was present between compared sequences.

Results

Sampling, screening and selection of best PHB producing bacterial strain

A total of 97 well formed bacterial colonies (10 from root nodules, 7 from rhizospheric soil sample, 10 from composting site soil, 4 from house-hold water, 7 from waste water sample, 12 from sewage sample, 12 from salty water sample, 16 from municipal waste soil, 9 from ground soil and 10 from mud soil sample) were selected, re-streaked on nutrient agar. To differentiate PHB producing bacteria from non PHB producing bacteria, carbol fuchsin staining and Sudan Black B staining was carried out. Out of a total of 97, 21 isolates were found having dark coloured granules of PHB within their cells with pink background after staining. All the selected 21 isolates and the standard strain were subjected to quantitative estimation of PHB by dispersion method. Highest PHB producing bacterial isolate was obtained from composting soil sample and that was identified as *Bacills megaterium* strain on the basic of morphological, biochemical and molecular characteristics. The complete results regarding these experiments can

be found out in our previous publication (Sharma and Dhingra 2015).

Development of mutant strains for increasing PHB production

Genetic improvements have proved to be the promising approach for increased production of desired product by industrially important microorganisms. The effect of mutation on *Bacillus megaterium* (M.Com2(1)) was investigated. The mutagens used were a physical mutagen viz. UV irradiation and chemical mutagen viz. Ethyl methanesulfonate (EMS).

UV mutagenesis**-** In this study, attempts were made to generate the mutants by subjecting the *Bacillus megaterium* (M.Com2(1)) to UV irradiation for different periods of time. The survival rate decreased with increasing in time of exposure to UV radiation (Tab. 6). Plate with minimum number of colony were selected for strain improvement because the fewer survivors in the sample would have undergone repeated or multiple mutations which may lead to the enhancement in the productivity of the culture. Minimum survival colonies were obtained with 5min UV exposure which resulted in the survival of four colonies, those were named as UV1, UV2, UV3 and UV4 (Fig. 1). These colonies were found positive for PHB production on nutrient agar plates with Sudan Black B staining (Fig. 2). These colonies were analyzed quantitatively in shake flasks for their potential for PHB production. Even though the time course study did not reveal any significant difference in the growth pattern of the mutant derivatives, when compared with the wild type, one mutant (UV2) showed a slight increase in the PHB production (Tab. 7). The UV2 mutant produced 24.70% (w/w) PHB yield, while the PHB yield of wild type was 23.97% (w/w). This showed that UV irradiation was not efficient in the improvement of *Bacillus megaterium* (M.Com2(1)) for increased PHB production.

Ethyl methanesulfonate (EMS) mutagenesis**-** The results obtained after the treatment of *Bacillus megaterium* (M.Com2(1)) with EMS for different exposure time are shown in table . The results showed that exposure of the bacterial strain to EMS produced a time-dependent killing of the strain, which increased gradually with the increase of exposure time. Cells treated with EMS for 45min resulted in the survival of 11 colonies and these were selected for the primary screening of PHB production on nutrient agar plates with Sudan Black B staining (Fig. 5) and then screened for PHB production. The screening studies resulted in two mutants, namely EMS7 and EMS8 and the mutant EMS7 was a better PHB producer compared with the wild type strain showed an increased PHB yield of 42.54% (w/w) (Tab. 9). The PHB yield of second mutant, EMS8, was 37.11% (w/w) (Fig. 6).

Isolation and characterization of PHB producing genes

PCR was used to amplify PHB biosynthesis genes such as *phaB* (Acetoacetyl CoA reductase) and *phaC* (PHA synthase) from the genomic DNA of both *Bacillus megaterium* (M.Com2(1)) and mutant strain and the results are described below:

Isolation and characterization of *phaB* gene- PCR was carried out to amplify the *phaB* gene from both wild type and mutant *Bacillus megaterium* (M.Com2(1)). The PCR products were run in an agarose gel along with the standard DNA marker, which showed the expected size of both the fragments (Fig. 7). The PCR amplicons were found to be 744bp of size. The *phaB* gene was sequenced completely and the sequence data was subjected to BLAST analysis. The BLAST results of the sequence showed high homology with acetoacetyl CoA reductase gene of *Bacillus megaterium* (M.Com2(1)). The amino acid sequence of this DNA fragment was continuous stretch of 247amino acids.

Isolation and characterization of *phaC* gene- PCR was carried out to amplify the *phaC* gene from both wild type and mutant *Bacillus megaterium* (M.Com2(1)). The PCR products were run in an agarose gel along with the standard DNA marker, which showed the expected size of both the fragments (Fig. 8). The PCR amplicons were found to be 1089bp of size. The *phaC* gene was sequenced completely and the sequence data was subjected to BLAST analysis. The BLAST results of the sequence showed high homology with PHA synthase gene of *Bacillus megaterium* (M.Com2(1)). The amino acid sequence of this DNA fragment was continuous stretch of 361 amino acids.

Comparative analysis between wild type and mutant with respect to PHB producing genes

Nucleotide sequence alignments were performed using the ClustalW program. Single nucleotide changes were identified in both the mutated *phaB* and *phaC* gene sequences as compare to wild type *phaB* and *phaC* gene sequences. Nucleotide base G is replaced by T in mutant as an effect of EMS. By observing the changes in wild type and mutant gene sequences and increased PHB production in mutant strain it might be hypothesized that changes may be the reason of increased PHB production (Fig. 9; Fig. 10).

Discussion

A developing environmental awareness has initiated a renewed scientific interest in PHB. PHB has physical and chemical properties very much similar to synthetic plastic. Hence, it can be used as an alternative of petroleum based synthetic plastic. However production costs are still a drawback to wider usage of these biopolymers (Keshavarz and Roy 2010). This study was an attempt to identify the native PHB producers with possible industrial applications. For this purpose, hydrocarbon rich soil, salty water and root nodules sample were selected. The use of Sudan Black B to determine the accumulation of PHB by bacteria was demonstrated (Hartman 1940; Iqbal et al. 2016).

Physical (UV light) and chemical (Ethyl methanesulfonate) mutagens were highly effective. The PHB yield varies considerably between the wild type strain and the mutant. The PHB yield increased from 23.97% (Wild type strain) to 24.70% (UV light mutant) and 42.54% (EMS mutant). The highest yield was observed in the chemical (EMS) mutagen. This indicated that for a significant increase in

PHB yield EMS could be a suitable mutagen. Moreover, it was reported that among the UV-mutants screening for high PHB granules were analyzed as compared to the parental strain (Sreeju et al. 2011; Pal et al. 2009). Both physical and chemical mutagens have been used to improve industrial strains. Hikmet and team have reported a significant increase in the PHB yield of *B. megaterium* Y6, *B. subtilis* K8 and *B. firmus* G2 through mutation (Hikmet et al. 2003). It was also reported that 70% (w/w) PHB in optimum culture conditions for *Bacillus* spp. IPCB-403 (Dave et al. 1996). Findlay and White (1983) reported the presence of PHB in *B. megaterium* chromatographically. Chen et al. (1991) studied D (-)-3-hydroxyalkanoate in 11 different *Bacillus* sp. and observed that 50% (w/w) PHB in the bacteria. Many of the *Alcaligenes eutrophus* strains used for PHB production on commercial scale have 96% (w/w) PHB (Bashir et al. 2014). A combination of genetic engineering and molecular microbiology techniques has been applied to enhance PHB production in microorganisms. Several genotypic methods applied various PCR protocols; using degenerate primers to detect and amplify the PHA synthase gene(s) have been reported (Solaiman et al. 2000; Sheu et al. 2000; Shamala et al. 2003; Solaiman and Ashby 2005; Chien et al. 2007; Berekaa and Al Thawadi 2012). PHB biosynthesis genes of wild type and mutant were identified and sequenced from *B. megaterium* (M.Com2(1)) wild type and its mutant. The sequences of *phaB* and *phaC* gene from wild type and mutant strain of isolate M.Com2(1) were deposited in GenBank under accession number KT725596, KU233682, KT725597 and KU233683 respectively. The *phaB* gene, which codes for NADPH dependent acetoacetyl CoA reductase was sequenced from isolate wild type and mutant M.Com2(1). The *phaB* gene sequences were complete ORF for 247 amino acids and the sequence was similar to other *Bacilli*, specially to that of *B. megaterium* (McCool and Cannon 2001). Indeed, the sequences of *phaC* synthase gene of wild type and mutant isolate M.Com2(1) revealed very close identity to other *phaC* gene sequences from other *Bacilli*. It showed 99% identity to *phaC* gene for polyhydroxyalkanoate synthase of *Bacillus megaterium* DSM319, *Bacillus megaterium* QMB1551, and 98% to synthase of *Bacillus megaterium* WSH-002 (Liu et al. 2011). The nucleotide sequence was a 1089 bp long complete ORF coding for a polypeptide of approximately 360 amino acids. In concurrence, successful application of different molecular approaches for detection of PHB accumulation by a variety of bacteria was reported by many scientists (Sheu et al. 2000; Chien et al. 2007; Castano et al. 2007). In this study, we compared wild type and mutant gene sequences of *phaB* and *phaC* genes by using ClustalW. Our data show that single nucleotide changes were present in the mutated gene sequences as compared with wild type gene sequences of *phaB* and *phaC* genes. To determine the variation in aligned sequences ClustalW was used (Nobrega and Nobrega 1986; Mathe et al. 2006; Keightley et al. 2009; Nordstrom et al. 2013). However, use of ClustalW for the identification of mutation in PHB producing gene has not been reported yet.

Tab. 1 Primers used for PCR detection of gene involved in PHB production

Primer	Sequence	Target gene	Expected Size (bp) of gene
PhaB(Forward)	5'GCAAGGGGGAAATTTTTCAT3'	*phaB*	755
PhaB(Reverse)	5'TATAAGCCGCCGTTAATGTT3'	*phaB*	756
PhaC(Forward)	5'AAGGGTGTGTATATTCGTGG3'	*phaC*	1100
PhaC(Reverse)	5'TTAGAGCGTTTTTCTAGCCA3'	*phaC*	1000

Tab. 2 Components of PCR reaction mixture for amplification of *phaB* gene

PCR component	Amount
Bacillus megaterium wild/ Mutant strain genomic DNA (A_{260}/A_{280}=1.8)	2µl
dNTP mix (250µM)	1.2µl
PhaB(forward) (20µM)	0.5µl
PhaB(Reverse) (20µM)	0.5µl
Taq polymerase (30U/50µl)	0.6µl
Buffer (1X)	1.5µl
$MgCl_2$ (2.5mM)	0.6µl
Nuclease free water	8.1µl

Tab. 3 The PCR reaction parameters for detection of *phaB* gene by specific primers

PCR Steps	Temperature	Time
Initial denaturation	94°C	1min
Denaturation	94°C	1min
Annealing	55°C	1min
Extension	72°C	2min
Final extension	72°C	5min
Hold	4°C	Infinite time

Tab. 4 Components of PCR reaction mixture for amplification of *phaC* gene

PCR component	Amount
Bacillus megaterium wild/ Mutant strain genomic DNA (A_{260}/A_{280}=1.8)	2µl
dNTP mix (250µM)	1.2µl
PhaC(forward) (20µM)	0.5µl
PhaC(Reverse) (20µM)	0.5µl
Taq polymerase (3U/50µl)	0.6µl
Buffer (1X)	1.5µl
$MgCl_2$ (2.5mM)	0.6µl
Nuclease free water	8.1µl

Tab. 5 The PCR reaction parameters for detection of *phaC* gene by specific primers

PCR Steps	Temperature	Time
Initial denaturation	94°C	1min
Denaturation	94°C	1min
Annealing	45°C	1min
Extension	72°C	2min
Final extension	72°C	5min
Hold	4°C	Infinite time

Tab. 6 Effect of UV irradiation on *Bacillus megaterium* (M.Com2(1))

Time of exposure to UV irradiation	CFU/ml	No. of survivors
0min	$120\times10^4\pm0.33$	120
0.5min	$43\times10^4\pm0.50$	43
1.0min	$23\times10^4\pm0.33$	23
2.0min	$16\times10^4\pm0.66$	16
5.0min	$4\times10^4\pm0.33$	4
10min	-	-
15min	-	-

Tab. 7 Comparison of PHB production of UV mutants with wild type

Strains	Biomass(g/l)	PHB(g/l)	PHB Yield% (w/w)
Wild Type	4.95±0.05	1.18±0.008	23.97±0.008
UV1	5.07±0.05	1.1±0.005	21.66±0.01
UV2	5.11±0.02	1.25±0.05	24.70±0.009
UV3	4.90±0.05	1.16±0.01	23.74±0.009
UV4	4.52±0.01	1.01±0.03	22.36±0.01

Tab. 8 Effect of Ethyl methanesulfonate treatment on *Bacillus megaterium* (M.Com2(1))

Time of exposure to EMS	CFU/ml	No. of survivors
0min	$150\times10^4\pm0.57$	150
5min	$130\times10^4\pm0.33$	130
15min	$117\times10^4\pm0.33$	117
30min	$63\times10^4\pm0.66$	63
45min	$11\times10^4\pm0.33$	11
60min	-	-

Tab. 9 Comparison of PHB production of EMS mutants with wild type

Strains	Biomass(g/l)	PHB(g/l)	PHB Yield% (w/w)
Wild Type	5.25±0.05	1.28±0.01	24.5±0.03
EMS1	4.87±0.01	1.13±0.01	23.19±0.01
EMS2	4.14±0.01	0.83±0.005	20.01±0.008
EMS3	5.15±0.01	1.46±0.005	28.30±0.02
EMS4	4.19±0.01	0.99±0.03	23.69±0.01
EMS5	4.9±0.005	1.47±0.006	30.23±0.01
EMS6	4.24±0.01	1.06±0.003	25.09±0.03
EMS7	4.93±0.03	2.11±0.01	42.54±0.007
EMS8	4.63±0.06	1.74±0.02	37.11±0.006
EMS9	3.97±0.006	0.89±0.003	22.64±0.007
EMS10	4.76±0.005	1.23±0.005	25.87±0.008
EMS11	4.98±0.003	1.30±0.003	26.31±0.007

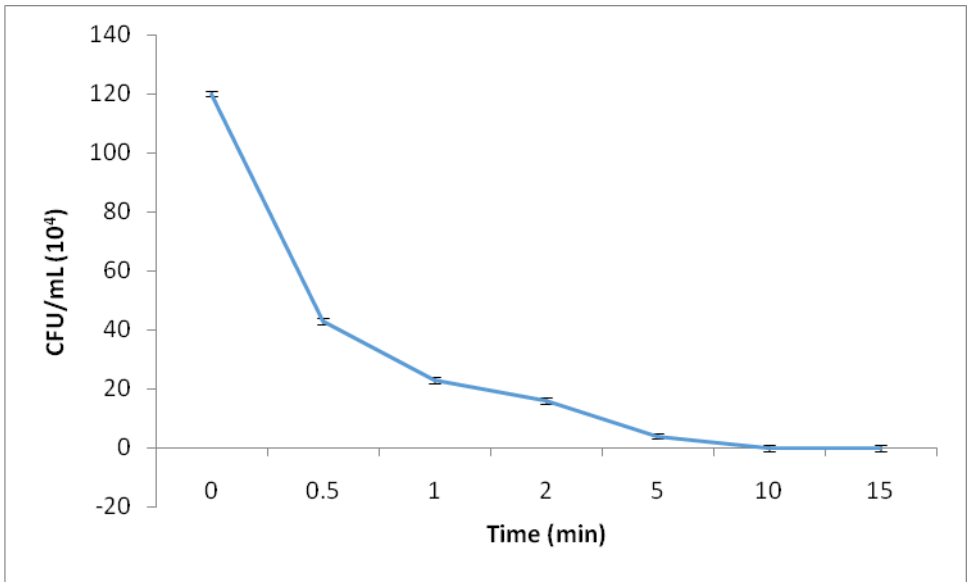

Fig. 1 Effect of UV irradiation on *Bacillus megaterium* (M.Com2(1))

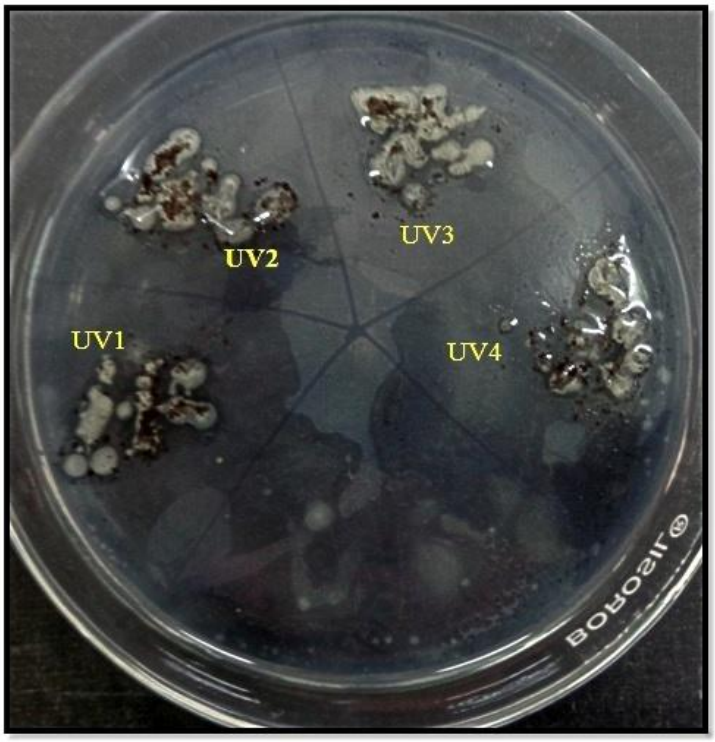

Fig. 2 Detection of PHB production by UV mutant strains of *Bacillus megaterium* (M.Com2(1)) based on Sudan Black B dye screening

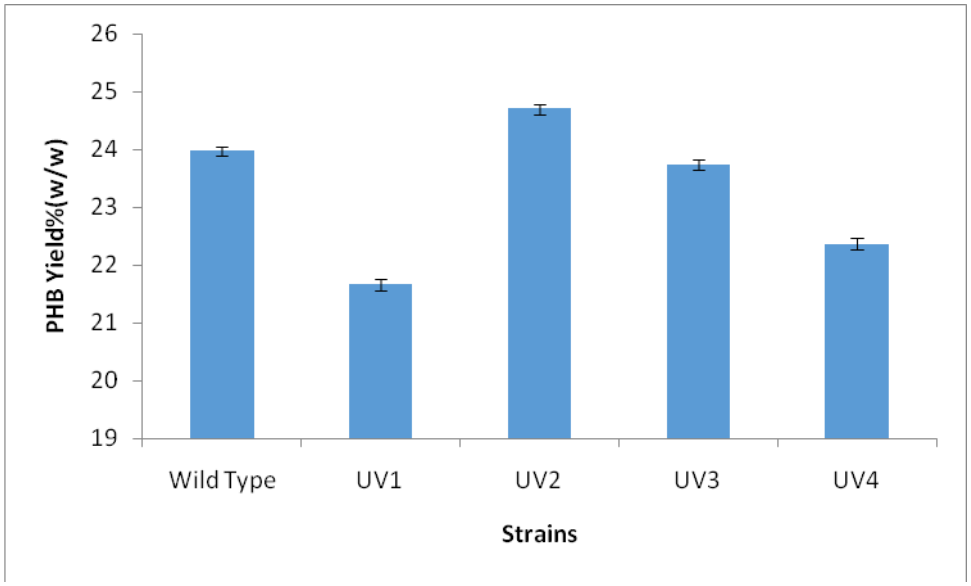

Fig. 3 Comparison of PHB production of UV mutants with wild type

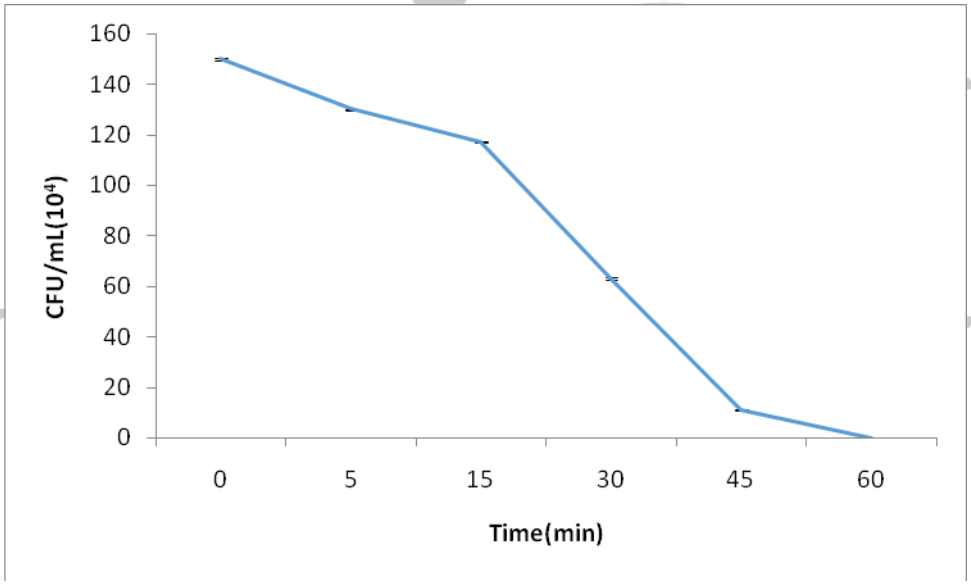

Fig. 4 Effect of ethyl methanesulfonate treatment on *Bacillus megaterium* (M.Com2(1))

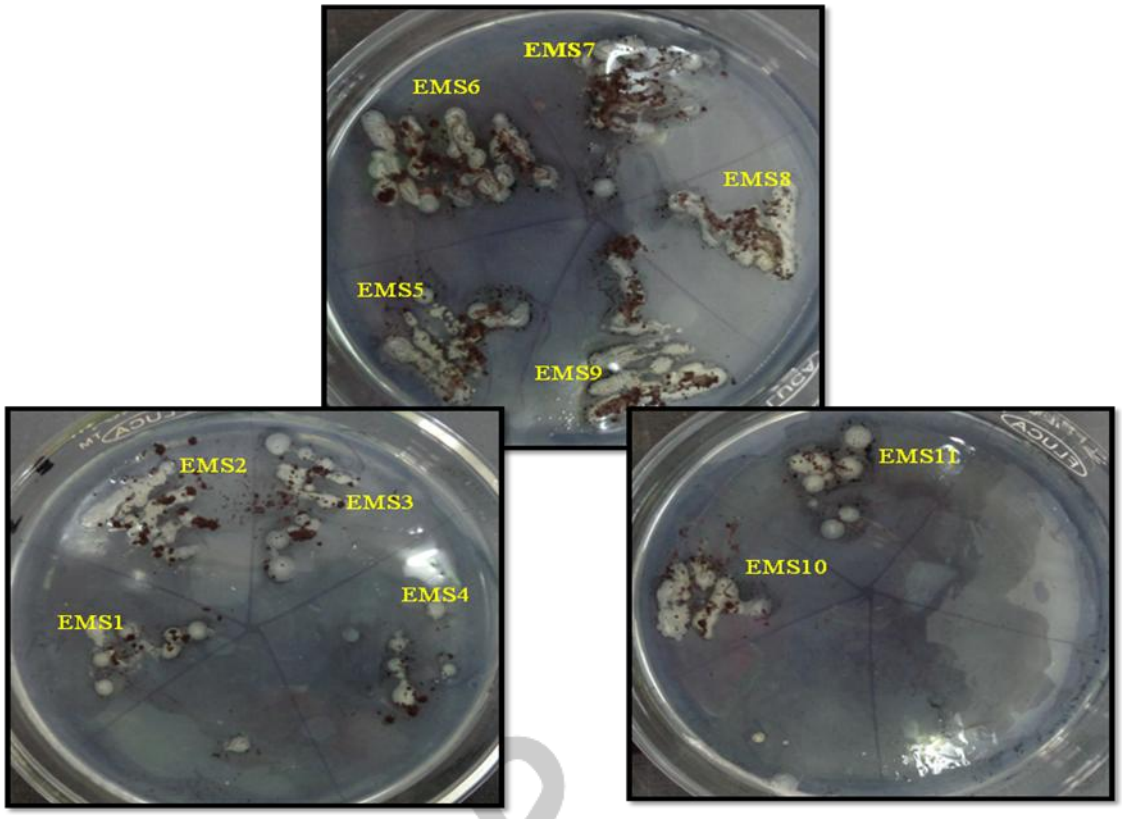

Fig. 5 Detection of PHB production by EMS mutant strains of *Bacillus megaterium* (M.Com2(1)) based on Sudan Black B dye screening

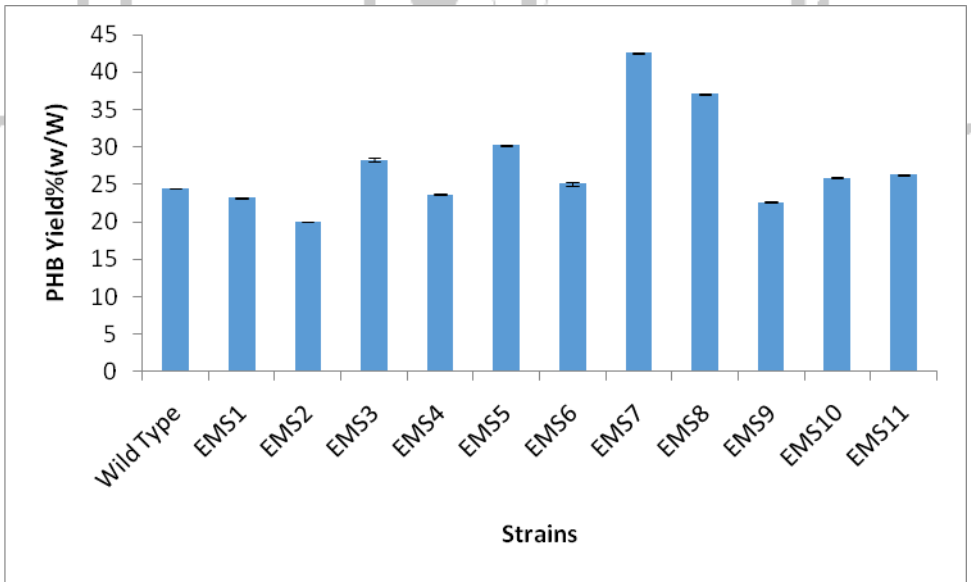

Fig. 6 Comparison of PHB production of EMS mutants with wild type

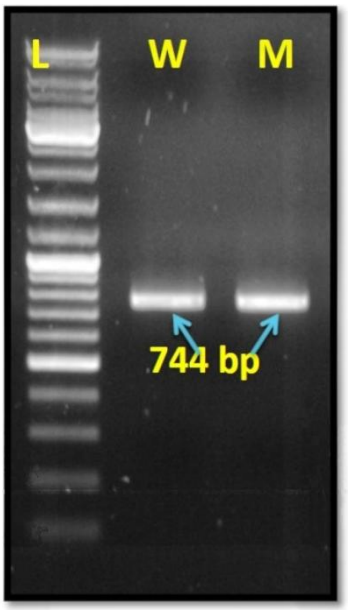

Fig. 7 PCR amplification of *phaB* gene of *Bacillus megaterium* (M.Com2(1)) wild type and mutant strain

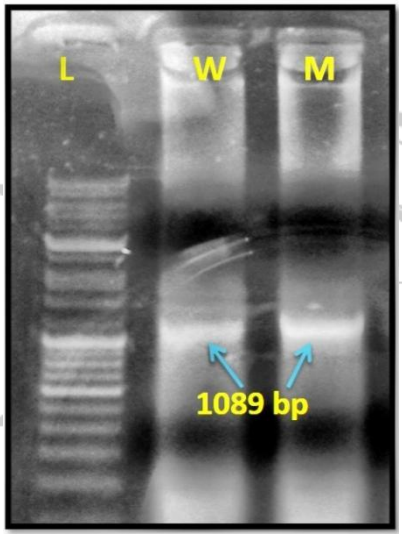

Fig. 8 PCR amplification of *phaC* gene of *Bacillus megaterium* (M.Com2(1)) wild type and mutant strain

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W ATGACAACATTACAAGGTAAAGTAGCAATCGTAACAGGCGGATCTAAAGGTATCGGGGCA
M ATGACAACATTACAAGGTAAAGTAGCAATCGTAACAGGCGGATCTAAAGGTATCGGGGCA
  ************************************************************
W GCAATTACACGTGAGCTTGCTTCTAATGGAGTAAAAGTAGCAGTAAACTATAACAGCAGT
M GCAATTACACGTGAGCTTGCTTCTAATGTAGTAAAAGTAGCAGTAAACTATAACAGCAGT
  *************************** ******************************
W AAAGAATCTGCAGAAGCAATTGTAAAAGAAATTAAAGACAACGGCGGAGAAGCTATTGCG
M AAAGAATCTGCAGAAGCAATTGTAAAAGAAATTAAAGACAACGGCGGAGAAGCTATTGCG
  ************************************************************
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W GTTCAAGCTGACGTGTCTTATGTAGATCAAGCAAAACACCTAATCGAAGAAACAAAAGCT
M GTTCAAGCTGACGTGTCTTATGTAGATCAAGCAAAACACCTAATCGAAGAAACAAAAGCT
  ************************************************************
W GCGTTTGGTCAATTAGACATTCTAGTAAACAATGCTGGAATTACGCGCGACCGTTCATTC
M GCGTTTGGTCAATTAGACATTCTAGTAAACAATGCTGGAATTACGCGCGACCGTTCATTC
  ************************************************************
W AAGAAGTTAGGTGAAGAAGATTGGAAAAAAGTAATTGATGTAAACTTACATAGCGTATAC
M AAGAAGTTAGGTGAAGAAGATTGGAAAAAAGTAATTGATGTAAACTTACATAGCGTATAC
  ************************************************************
W AACACAACATCAGCTGCGCTAACGCACCTTTTAGAATCTGAAGGTGGTCGTGTTATCAAT
M AACACAACATCAGCTGCGCTAACGCACCTTTTAGAATCTGAAGGTGGTCGTGTTATCAAT
  ************************************************************
W ATTTCATCAATTATTGGTCAAGCGGGCGGATTTGGTCAAACAAACTACTCAGCTGCTAAA
M ATTTCATCAATTATTGGTCAAGCGGGCGGATTTGGTCAAACAAACTACTCAGCTGCTAAA
  ************************************************************
W GCAGGTATGCTAGGATTCACTAAATCATTAGCTCTTGAGCTAGCTAAAAGTGGCGTAACG
M GCAGGTATGCTAGGATTCACTAAATCATTAGCTCTTGAGCTAGCTAAAAGTGGCGTAACG
  ************************************************************
W GTTAATGCAATTTGCCCAGGATTTATTGAAACGGAAATGGTAATGGCAGTGCCGGAAGAA
M GTTAATGCAATTTGCCCAGGATTTATTGAAACGGAAATGGTAATGGCAGTGCCGGAAGAA
  ************************************************************
W GTACGTGCAAAAGTTGTAGCAAAAATCCCAACTCGTCGCTTAGGCCACGCTGAAGAAATT
M GTACGTGCAAAAGTTGTAGCAAAAATCCCAACTCGTCGCTTAGGCCACGCTGAAGAAATT
  ************************************************************
W GCACGTGGAGTTGTTTACTTAGCAAAAGACGGCGCGTACATTACAGGACAACAGTTAAAC
M GCACGTGGAGTTGTTTACTTAGCAAAAGACGGCGCGTACATTACAGTACAACAGTTAAAC
  ******************************************** ************
W ATTAACGGCGGCTTATACATGTAA
M ATTAACGGCGGCTTATACATGTAA
  ************************
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Fig. 9 Alignment *phaB* gene sequence of wild type and mutant strain with ClustalW

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W GTGGCAATTCCTTACGTGCAAGAGTGGGAAAAATTAATCAAATCAATGCCAAGTGAATAT
M GTGGCAATTCCTTACGTGCAAGAGTGGGAAAAATTAATCAAATCAATGCCAAGTGAATAT
  ************************************************************
W AAAAGTTCTGCAAGACGTTTTAAGCGTGCATATGAAATTATGACAACAGAAGCGGAACCG
M AAAAGTTCTGCAAGACGTTTTAAGCGTGCATATGAAATTATGACAACAGAAGCGGAACCG
  ************************************************************
W GAAGTTGGATTAACACCAAAAGAGGTTATTTGGAAAAAGAACAAAGCAAAATTATACCGC
M GAAGTTGGATTAACACCAAAAGAGGTTATTTGGAAAAAGAACAAAGCAAAATTATACCGC
  ************************************************************
W TATACGCCAGTAAAAGATAACCTGCATAAAACACCAATCTTACTCGTATATGCATTGATC
M TATACGCCAGTAAAAGATAACCTGCATAAAACACCAATCTTACTCGTATATGCATTGATC
  ************************************************************
W AATAACCTGTATATTTTGGATTTAACACCTGGAAACAGCCTTGTTGAATACTTATTAAAC
M AATAACCTGTATATTTTGGATTTAACACCTGGAAACAGCCTTGTTGAATACTTATTAAAC
  ************************************************************
W CGCGGTTTTGACGTATATTTGCTTGACTGGGGAACTCCTGGGCTTGAAGACAGCAATATG
M CGCGGTTTTGACGTATATTTGCTTGACTGGGGAACTCCTGGGCTTGAAGACAGCAATATG
  ************************************************************
W AAGCTAGATGATTATATTGTAGATTATATTCCAAAAGCGGCGAAAAAGGTGCTGCGCACT
M AAGCTAGATGATTATATTGTAGATTATATTCCAAAAGCGGCGAAAAAGGTGCTGCGCACT
  ************************************************************
W TCTAAATCTCCTGATTTGTCTGTTCTTGGTTACTGCATGGGCGGAACTATGACATCTATT
M TCTAAATCTCCTGATTTGTCTGTTCTTGTTTACTGCATGGGCGGAACTATGACATCTATT
  *************************** ******************************
W TTTGCTGCATTAAATGAAGACTTGCCGATTAAAAACTTAATTTTTATGACAAGTCCATTT
M TTTGCTGCATTAAATGAAGACTTGCCGATTAAAAACTTAATTTTTATGACAAGTCCATTT
  ************************************************************
W GATTTTTCGGATACAGGTTTATACGGAGCATTTTTAGACGATCGCTACTTTAATTTAGAT
M TATTTTTCGGATACAGGTTTATACGGAGCATTTTTAGACGATCGCTACTTTAATTTAGAT
   ***********************************************************
```

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W AAAGCAGTAGATACATTCGGAAACATCCCTCCAGAGATGATTGACTTTGGAAACAAGATG
M AAAGCAGTAGATACATTCGGAAACATCCCTCCAGAGATGATTGACTTTGGAAACAAGATG
  ************************************************************

W TTAAAGCCAATCACGAATTTCTATGGCCCATATGTAACGTTGGTGGACCGTTCGGAAAAT
M TTAAAGCCAATCACGAATTTCTATGGCCCATATGTAACGTTGGTGGACCGTTCGGAAAAT
  ************************************************************

W CAGCGGTTTGTTGAAAGCTGGAAGCTAATGCAAAAGTGGGTTGCTGACGGAATCCCATTT
M CAGCGGTTTGTTGAAAGCTGGAAGCTAATGCAAAAGTGGGTTGCTGACGGAATCCCATTT
  ************************************************************

W GCTGGTGAAGCTTATCGTCAGTGGATTCGTGACTTCTATCAACAAAATAAATTAATCAAT
M GCTGGTGAAGCTTATCGTCAGTGGATTCGTGACTTCTATCAACAAAATAAATTAATCAAT
  ************************************************************

W GGTGAACTTGAAGTTCGCGGACGCAAAGTAGATTTGAAAAATATTAAAGCTAATATTTTA
M GGTGAACTTGAAGTTCGCGTACGCAAAGTAGATTTGAAAAATATTAAAGCTAATATTTTA
  ****************** ***************************************

W AACATTGCTGCTAGCCGTGATCATATTGCGATGCCACATCAAGTGGCAGCATTAATGGAC
M AACATTGCTGCTAGCCGTGATCATATTGCGATGCCACATCAAGTGGCAGCATTAATGGAC
  ************************************************************

W GCTGTTTCAAGTGAAGATAAAGAGTATAAATTGTTGCAAACAGGTCACGTATCTGTTGTA
M GCTGTTTCAAGTGAAGATAAAGAGTATAAATTGTTGCAAACAGGTCACGTATCTGTTGTA
  ************************************************************

W TTTGGTCCAAAAGCAGTGAAGGAAACATACCCTTCAATCGGCGATTGGCTAGAAAAACGC
M TTTGGTCCAAAAGCAGTGAAGGAAACATACCCTTCAATCGGCGATTGGCTAGAAAAACGC
  ************************************************************

W TCTAAATAA
M TCTAAATAA
  *********
```

Fig. 10 Alignment *phaC* gene sequence of wild type and mutant strain with ClustalW

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Conflict of interest statement

The authors declare no commercial or financial conflict of interest.

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