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Enhancement of Commercial Production of Polymeric PHB Material from Bacterial Strains through Mutagenic Strategies

Madhuri Girdhar[1], Ajay Sharma[2] and Anand Mohan[1]

[1]Department of Biotechnology, Lovely Professional University, Punjab, India.
[2]Department of Chemistry, Lovely Professional University, Punjab, India.

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Poly-β-Hydroxybutyrate (PHB) is proving itself as an advance polymeric material as well as an alternative for plastic material. The only hinge stopping large scale utilization of PHB at industrial scale and in daily lives is its, economical production. Large scale economical production of PHB depends upon optimization and selection of high PHB producing strains. The present study investigates mutagenic strategies for the improvement of commercial strains for high scale PHB production in industries. Improvement of strains by mutation is very cost effective method and strategically better approach for getting maximum output of PHB. The mutation treatment was performed by Physical and Chemical methods i.e. Ultra violet radiation (Physical) and Acridine Orange treatment (Chemical). Mutation leads to an increase in the yield of PHB as in comparison to original strains which has proved through increase in PHB extraction from the mutated counterparts of *Bacillus flexus* and *Bacillus megaterium*. The results revealed that there was an excellent improvement in PHB production by almost 2 folds, when strain treatment was performed with physical mutagen (UV radiation) but no such improvement was seen with chemical mutagen (Acridine Orange). The PHB yield increased marginally from 15.68% to 18.61% with chemical mutagen where as in UV treatment the PHB yield increased exceptionally, from 15.68% to 23.70% in case of *Bacillus flexus*[Agriculture isolate M1] . In case of *Bacillus megaterium* UV exposed strain show a phenomenal increase in PHB yield from 11.76% to 25% but no such improvement again was seen in PHB yield through Acridine orange treated colonies i.e. 11.76% to 15.09%. The quality of produced PHB was assessed through FTIR. These results exemplify that physical mutagens can be more effective for improving commercial production of PHB from bacterial strains.

Key words: Poly-β-Hydroxybutyrate, mutagen, FTIR, economical.

Poly-3-hydroxybutyrate (PHB) is a linear polyester of D (-)-3 hydroxybutyric acid and is a hotspot material for researchers and scientists working in the field of material and polymer science[1]. It was first discovered in bacteria by Lemoigne in 1925[1]. Polyhydroxyalkanoates (PHAs) are the major series of polyester polymer under which PHB falls and these are synthesized by various microorganisms such as *Pseudomonas putida*, *Alcaligenes latus*, *Aeromonas hydrophila*, *Bacillus* spp. and *Ralstonia eutropha* [2]. The condition of nutrient limitation other than carbon source[3] or starvation causes the generation and accumulation of PHB in the form of intracellular granules by wide variety of Gram-positive and Gram-negative organisms. Now a days, Global

* To whom all correspondence should be addressed.
E-mail: anandmohan77@gmail.com

environmental concerns and solid waste management problems have generated considerable interest in the development of biodegradable plastics with desired physical and chemical properties similar to conventional synthetic plastics. The plastics derived from petrochemicals take millions of years to deplete so to replace this synthetic plastic, biodegradable plastic is required. PHB is a potential candidate for replacement of synthetic plastics as this is a biodegradable and environment friendly plastic[4]. PHB has found a wide range of applications, as for producing films, bottles, fibres and mulch materials for agriculture. PHB usage is not limited in these areas only but is also utilized in biomedical areas such as for osteo-synthetic materials, bone plates, surgical sutures and many other biomedical materials[5]. Wide-spread substitution of conventional plastics has been limited by high production costs of PHB, Therefore more efforts ared needed to be devoted for making this process economically feasible by increasing our understanding of the PHB accumulation process and improving its productivity. In biotechnological terms, cheap substrates, mutations, and genetically modified high PHB-yielding bacteria (or plants) can be used for bio-polymer production[6]. "Mutagenesis" is the source of all genetic variations. Strain improvement is an essential part of process development for microbial products[7,8]. Strain improvement can be carried out by such techniques as rational screening and genetic engineering or by the traditional methods of mutagenesis and selection on the basis of direct titer measurement. Mutagenesis and screening method is a reliable and cost effective procedure. Various mutagens like Ultraviolet rays (UV), Acridine Orange are used to mutate some bacterial strains for the production of PHB[9,10]. Since this method is cost effective it can lead to the production of PHB at lower cost and increase in production levels. Various microbial strains when mutated by these mutagens can lead to the enhanced production of PHB leading to an overall decrease in cost [11].

Material and Methodology

Sample collection and Isolation of bacterial strains

Microbial strains were isolated from different samples collected from agriculture soil of Punjab (Majha Region) by the streak plate method on DSC-97 and nutrient agar media containing different salt concentrations.

Standard microbial strain MTCC 453 (*Bacillus megaterium*) was procured from IMTECH Chandigarh for mutagenesis experiment.

Media preparation

For microbial strains DSC-97 and nutrient agar media was prepared. The composition of DSC-97 in 1 litre of distilled water is as follow:- Peptone 7.5g, yeast extract 10g, trisodium citrate 3g, KCl 2 g, $MgSO_4 \cdot 4H_2O$ 20g, $FeCl_2$ 0.023 g , Agar 20 gram and pH 7.2[12].

Screening of PHB production by Sudan black

The strains were tested for the accumulation of PHB by Sudan black staining. Dye solution is prepared by adding 0.3 g of Sudan Black dye in 90% ethanol. Smear was prepared and heat fixed, further Sudan black is added over the smear for 15 minutes; wash the smear with distilled water and counter-stain with safranin. Slides were observed under microscope at 100X [Magnus Live (Olympus) Microscope]. Bluish- black spots inside the cell wall of organisms indicate the presence of PHB granules in the bacterial cell wall[13].

Improvement of the microbial strains by inducing Mutations by various agents

Mutations were induced for the improvement of microbial strains for increasing the production of PHB by different physical and chemical mutagenic agents. Physical and chemical mutagen utilized for the current experiments were Ultraviolet radiation and Acridine orange.

Inducing mutation by Physical mutagen

Preparation of Control sample

Overnight grown bacterial culture broth 20 ml was centrifuged at 10,000 rpm for 15 minutes at room temperature. Supernatant was discarded and pellet was taken and re-suspended in 1ml sterile saline. Sterile saline was prepared by 7.4 gm NaCl per litre distilled water. Pellets in sterile saline were further diluted at 10^{-1}, 10^{-2}, 10^{-3}, and spread on NA media plates. Incubation was done for 48 hours at 37^0 C. Total viable count (TVC) was measured[8,9,10].

Mutagenesis Experiment by UV radiation

10 ml bacterial broth culture was transferred to 3 test tubes. Expose these test tubes to ultraviolet light at 254 nm for different time intervals (1 minute, 5 minutes and 10 minutes). After exposure incubation was done by placing the test tubes in test tube box in dark and covered with

black cloth for 2-6 hours depending upon the growth curve. Serial dilution of mutant in subsequent generation of 10^{-1}, 10^{-2} and 10^{-3} were prepared. Out of this 0.1 ml was taken and spread over NA media and incubated at 37^0C for 48 hours. Total viable count was measured[8,9,10].

Inducing mutation by Acridine Orange

Acridine orange was used as chemical mutagen for inducing mutations for improvement of PHB production of microbial strains.

Preparation of Control sample

Overnight grown bacterial broth was taken and serial dilutions were prepared, further 0.1 ml of bacterial culture was taken and spread on NA media. Incubation was performed at 37^0C for 48 hours. Total viable colonies were counted.

Mutagenesis Experiment by Acridine orange

Quantity of 10 ml of bacterial broth in 3 test tubes was taken and Acridine orange solution was prepared by adding 0.1 gm Acridine orange in 10 ml distilled water. Add different concentrations; 10 µl, 50 µl and 100 µl of acridine orange in all test tubes. Incubate test tubes for 2-6 hours in dark place by covering it with black cloth. After incubation serial dilutions upto 10^{-1}, 10^{-2} and 10^{-3} were prepared and 0.1 ml of culture was taken and spread over NA media. Incubate the culture for 48 hours at 37^0C. Further total viable colony count was measured[8,9,10].

Extraction of PHB

Extraction of PHB was performed by chloroform extraction method from the mutated strains and wild strain. After incubating the culture at 37^0C for 48 hours, centrifuge the bacterial culture at 8000 rpm for 15 minutes. Supernatant was discarded and pellet was dissolved in sodium hypochlorite at 37^0C for 1-2 hours. Further centrifugation at 8000 rpm for 8 minutes was performed. Pellet was washed with distilled water, acetone and ethanol. The pellet was dissolved in boiling chloroform and allowed to evaporate overnight at room temperature to obtain crystals of PHB[14,15].

Cell dry weight

Cell dry weight determination was done with centrifugation of the culture medium. The supernatant was discarded and pellet was washed with distilled water and dried at 30^0C for 2 hrs. Dry weight was estimated by drying the pellet and weighing it.

Determination of PHB

Identification of PHB was done by thin layer chromatography (TLC). The mixture of silica gel (8.5 g), calcium sulphate (1.5 g) and distilled water (20 ml) was poured on glass plate and dried in oven for 3-4 hours. 0.4 gm of PHB was dissolved in chloroform and sample was loaded on TLC plate. Incubation of silica plate in benzene and ethyl acetate chamber in the ratio 1:1 for 30 minutes was carried out. Staining was performed with vapour of iodine solution (20ml) at 80-90^{0}C in water bath after running of PHB in the silica plate. Staining causes green-black colored spots after a time lag of around 15 minutes on TLC plate. Spot indicates the presence of PHB compound[5].

Characterization of PHB

There are various techniques used for characterization of PHB i.e. FTIR and GC/MS. FTIR technique was used for the initial identification of PHB sample. 1 mg of PHB and 10 mg of spectral pure anhydrous potassium bromide crystal was mixed for IR analysis. The relative intensity of transmitted light energy was measured against the wavelength of absorption for the region[16]. The Shimadzu FTIR facility in chemistry department of Lovely professional university was used for identification. The JEOL GC Mate instrument [SAIF Labs] Chandigarh, was used to identify the molecular mass of extracted compound, in which chloroform was used as solvent[17].

16 s RNA sequencing for characterization of bacteria

Identification of isolated bacteria was analysed by 16s rRNA sequencing and was done at Yaazh Xenomics, Madurai.

RESULTS AND DISCUSSION

Sample collection and Isolation of microbial strains from Agriculture Land

Soil samples were collected from agriculture sites (Majha region) of Punjab. Soil sample was taken and serial dilutions of 10^{-6}, 10^{-7} and 10^{-8} were prepared and spread on NA media plates. Petri plates were incubated at 37^0C for 48 hours. Growth was observed and single specific colonies were streaked on DSC-97 and nutrient agar media and incubated for 2 days at 37^0C. Standard microbial strain *Bacillus megaterium*

(MTCC No.453) was utilized for comparison purpose and was also grown on NA media plates.

Screening of PHB production by Sudan black

Strains were stained with Sudan black B for screening of PHB granules present in the cell wall of the bacterium. Slides were observed under 100X magnification of microscope (Fig 1 and Fig 2).

Growth curve of strains at different Incubation time

Isolated strains were grown on NA media and best growth of different strains were observed

Number of colonies in control & mutated samples after Exposure to UV light and Acridine Orange mutagen (Agriculture isolate M1)

Table 1. UV exposure and Total viable count of Agriculture isolate [M1]

	Duration of UV exposure and Total viable count			
Dilutions	Control	1 minute	5 minutes	10 minutes
10^{o}	56	28	15	6
10^{-1}	25	9	4	2
10^{-2}	17	2	-	-
10^{-3}	9	-	-	-

Table 2. Acridine orange treatment and Total viable count of Agriculture isolate [M1]

	Different concentration of Acridine Orange (µg/ml) and Total viable count				
Dilutions	Control	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
10^{o}	67	39	22	9	2
10^{-1}	35	17	4	1	-
10^{-2}	18	-	-	-	-
10^{-3}	10	-	-	-	-

Number of colonies in control & mutated samples after Exposure to UV light and Acridine Orange mutagen [*Bacillus megaterium* (MTCC 453)]

Table 3. UV exposure and Total viable count of *Bacillus megaterium* [MTCC453]

	Duration of UV exposure and Total viable count			
Dilutions	Control	1 minute	5 minutes	10 minutes
10^{o}	72	36	18	5
10^{-1}	47	21	4	2
10^{-2}	22	12	1	-
10^{-3}	14	7	-	-

Table 4. Acridine orange treatment and Total viable count of *Bacillus megaterium* [MTCC453]

	Different concentration of Acridine Orange (µg/ml) and Total viable count				
Dilutions	Control	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
10^{o}	76	29	13	7	2
10^{-1}	35	17	9	3	1
10^{-2}	21	9	2	-	-
10^{-3}	10	5	1	-	-

Table 5. PHB production by wild and mutant strain [Agriculture isolate (M1)]

Cell dry weight, PHB, PHB yield (%) of wild and mutated strain M1			
	Cell dry weight (g/l)	PHB (g/l)	PHB yield (%)
Pre-mutation (Control)	0.58	0.091	15.68
UV mutation	0.81	0.192	23.70
Acridine Orange mutation	0.65	0.121	18.61

Table 6. PHB production by wild and mutant strain [MTCC453]

Cell dry weight, PHB, PHB yield (%) of wild and mutated strain M1			
	Cell dry weight (g/l)	PHB (g/l)	PHB yield (%)
Pre-mutation (Control)	0.51	0.060	11.76
UV mutation	0.36	0.090	25
Acridine Orange mutation	0.53	0.080	15.09

by taking OD at 600 nm. Best growth of Agriculture isolate M1 was observed at incubation time of 72 hrs and *Bacillus megaterium* strain with an incubation time of 96 hrs (Figure 3 and 4).

The PHB yield in both the wild strain and mutated strain were analysed by measuring the Cell dry weight (cdw) and yield of extracted compound i.e. PHB. It was analysed that the wild strain i.e. M1 has cdw of 0.58 g/l and yield of 15.68% of the culture medium, where as its exposure to the physical mutagen i.e. UV increased the cdw from 0.58g/l to 0.81g/l and PHB yield from 15.68% to 23.70%. The chemical mutagen i.e. Acridine orange exposure to the strain M1

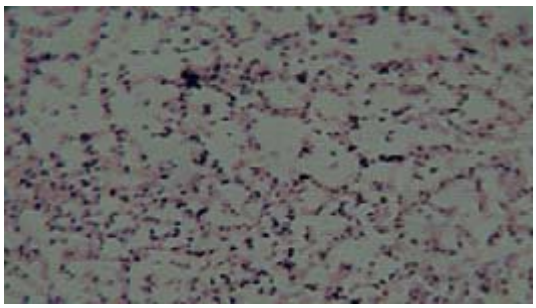

Fig. 1. Agriculture Isolate M1

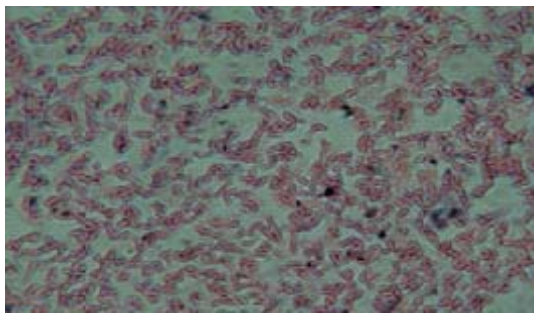

Fig. 2. *Bacillus megaterium* MTCC 453

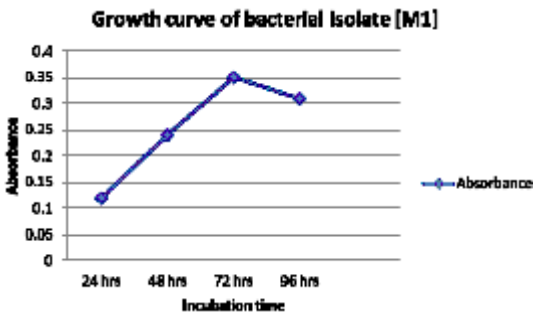

Fig. 3. Growth curve of bacterial isolate [M1] at different incubation time

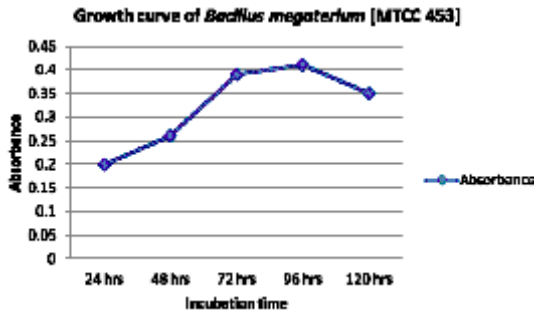

Fig. 4. Growth curve of *Bacillus megaterium* [MTCC 453] at different incubation time

increased the cdw from 0.58g/l to 0.65g/l, whereas the PHB yield increased from 15.68% to 18.61%. These results show that UV is a better mutagen for increasing the PHB yield (Table 5 and Figure 6).

The PHB yield in both the wild strain and mutated strain were analysed by measuring the Cell dry weight (cdw) and yield of extracted compound i.e. PHB. It was analysed that the wild strain i.e. *Bacillus megaterium* [MTCC453] has cdw of 0.51g/l and yield of 11.76%, where as its exposure to the physical mutagen i.e. UV increased the cdw from 0.51g/l to 0.36g/l and PHB yield from 11.76% to 25%. The chemical mutagen i.e. Acridine orange exposure to the strain M1 increased the cdw from 0.51g/l to 0.53g/l, whereas the PHB yield increased from 11.76% to 15.09%. These result shows that UV is a better mutagen for increasing the PHB yield (Table 6 and Figure 7).

Fig. 5. Extracted PHB by Solvent Extraction method

FTIR analysis

IR study was carried out for PHB produced from both the mutated and wild strains, the peak values observed for the PHB of isolate sp. was 1735.99cm^{-1}, 1460.16 cm^{-1} and 1261.49 cm^{-1} (wild) and 1737.92 cm^{-1}, 1458.23 cm^{-1} and 1163.11 cm^{-1} (mutated) which was near to the peak value of pure PHB (1726.03 cm^{-1} for C=O, 1286.65 for C-O and 1460.26 for C-H). So this data indicates that the isolated compound could be PHB (Figure 8,9a and 9b) .

TLC analysis of extracted PHB

PHB sample was determined by TLC. The occurrence of brown spot showed presence of PHB. Rf value was 0.76 (Figure 10).

The extracted compound was analysed by mass spectroscopy to evaluate the molecular

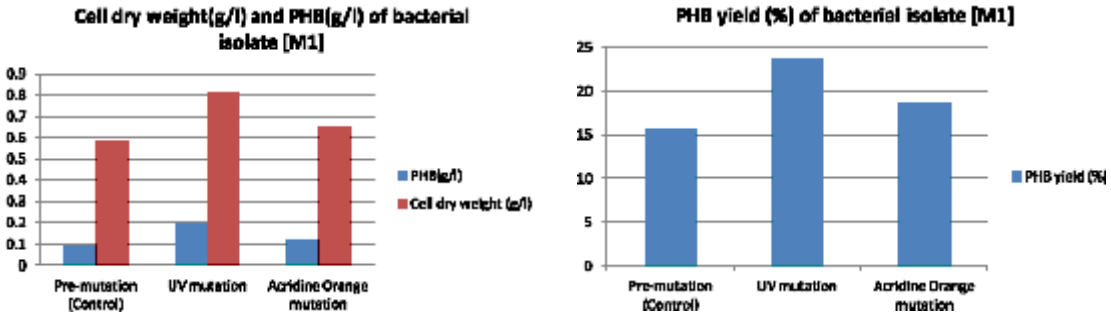

Fig. 6. PHB, DCW and PHB yield of isolated strain [Agriculture isolate M1]

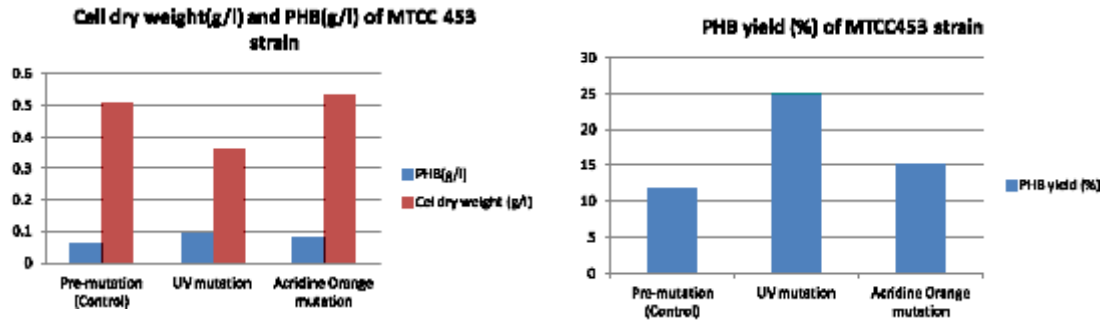

Fig. 7. PHB, DCW and PHB yield of standard strain [MTCC 453]

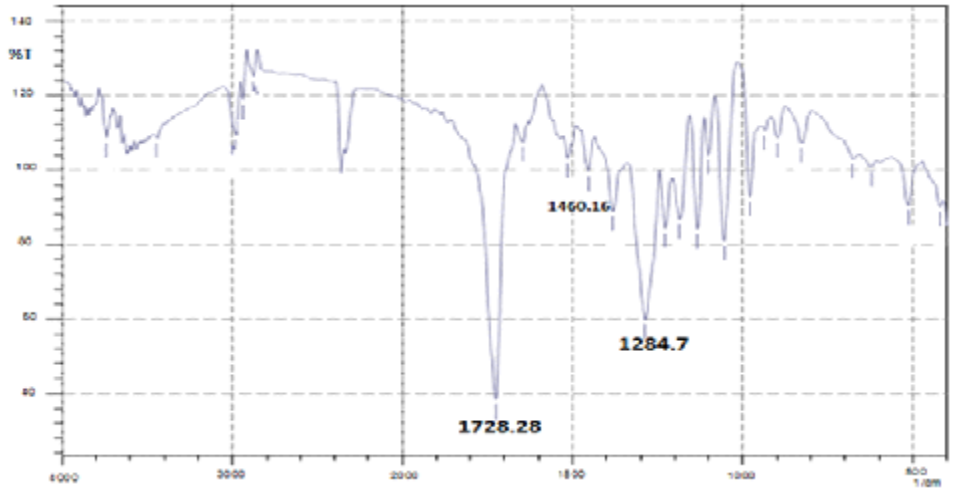

Fig. 8. FTIR analysis of control PHB (Sigma Aldrich)

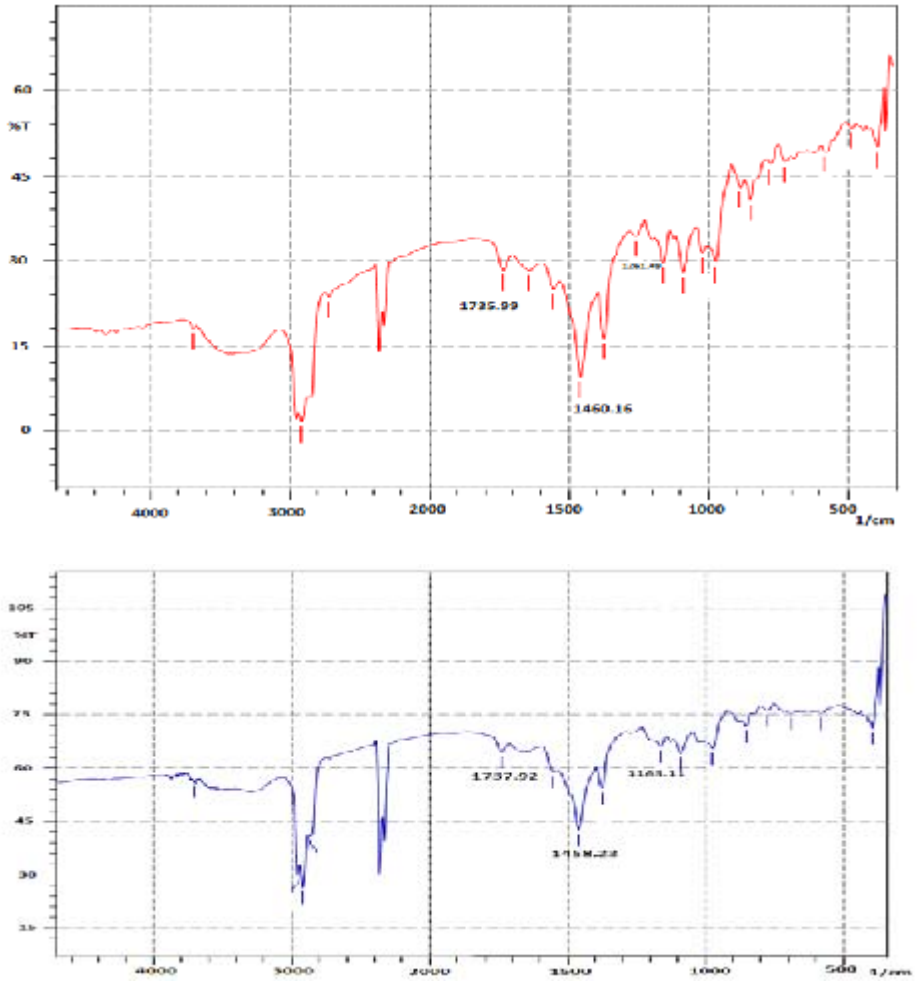

Fig. 9. FTIR analysis of extracted PHB (wild) [a] and mutated strain [b]

Fig. 10. Thin Layer Chromatography of extracted compound

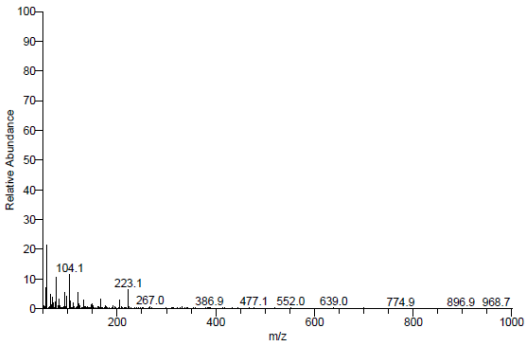

Fig. 11. The extracted sample is analysed by MASS SPECTROSCOPY

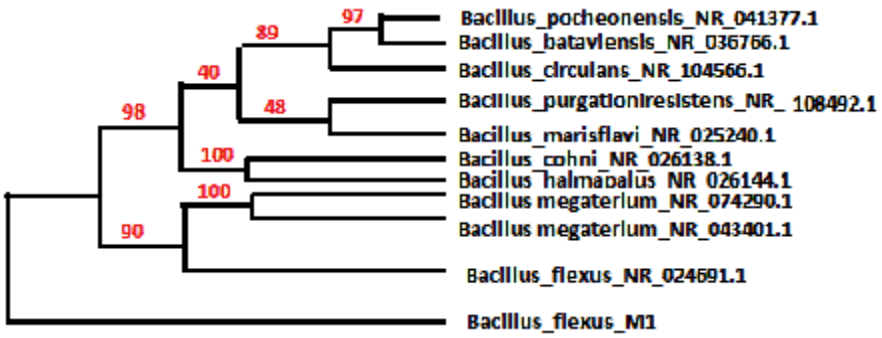

Fig. 12. Phylogenetic tree of M1 isolate

weight of the extracted samples. The results showed the peak at m/z 104.1 which was near to the peak of pure PHB (procured from Sigma Aldrich). The other small peaks observed below m/z 104.1 and at m/z 223.1 are the peaks of other homo-polymers and co-polymers of poly hydroxybutyrate (PHB) (Figure 11)

16s RNA for identification of isolate

The isolated bacterial diversity was analyzed by molecular approach i.e. 16s rRNA. In 16s rRNA sequence analysis, more than 99% similarity was observed between the sequences of the isolated strain and the sequences of *Bacillus flexus* M1 in Genbank and accession no. alloted was KJ939621 (Figure 12).

CONCLUSION

PHB is a highly strategic material due to its various properties, degradation values, biological origin and biocompatibility. All over the world researchers are continuously striving to increase its production from the existing bacterial strains as well from the new strains. Mutations can cause improvement in the production of PHB, If they occur in the relevant operator and promoter region of the operon causing the expression of proteins involved in production of PHB inside the cell. The current work dealt with inducing mutations (Physical (UV) and Chemical (AO) mutagenesis) in standard strain already a known PHB producer (MTCC 453) and strain isolated from agriculture soil of Punjab producing PHB. Mutated strains finally isolated in the current work were observed having an increase in the yield of PHB as compared to the wild strains. The FTIR analysis proved that the extracted material is PHB. The results of mass spectroscopy also revealed that the extracted material is PHB through analysis of its molecular weight.

The result of the current work proved that UV mutagen can prove better mutagen for causing

modifications at genomic levels and hence inducing the high PHB production as an intracellular granule inside the cell in mutated organism. The analysis also shows that UV mutant and Acridine orange mutant could be enhancing the activity of genes *pha a*, *pha b* and *pha c* encoding specific proteins and leading to the production of enhanced amount of PHB. These studies prove that mutagenesis can be utilized as specific strategy for enhancing the production of PHB. Further the mutated strains could easily be utilized for PHB production in specific commercial settings.

PHB is biodegradable plastic which is replacing fast, petroleum derived plastic. As with changing lifestyles now a days there is an increase in the demand of the plastic in daily use hence PHB can easily fill that gap as its biodegradable nature and other friendly properties could serve that purpose.

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