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Polyhydroxybutyrate (PHB) Production and Mutagenesis of Halophile isolates from the East African Rift Salt Lake

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Polyhydroxybutyrate are considerably of human interest due to their outstanding properties as bioplastic to effectively replace polyethylene. In this study, halophiles were used for the production of bioplastics. Current studies investigated the isolation of halophilic bacteria from the samples of Lake Natron, one of the salt lakes of the East African Rift Valley. Halobacterium medium was used for the isolation of the strains at 37°C and pH 8.0. A strategical approach for high PHB production was implemented in which high carbon source in the form of dextrose was given to the bacteria at regular intervals, without increasing the nitrogen source. Further studies were carried out on random mutagenesis approach [physical (UV) and chemical (Acridine orange)] for enhancing the production of PHB. The PHB was extracted using sodium hypochlorite digestion process followed by chloroform extraction technique. PHB was identified by Thin Layer Chromatography, FTIR and NMR techniques. Among the mutants, UV-mutant produced the highest PHB yield of 29.63% compared to the wild type whose PHB yield was 20%. Acridine orange mutant showed an increased PHB yield from 20% to 28%. 16s rRNA sequence of the strain1 was carried out and the Bacterium was identified as *Virgibacillus marismortui*.

Key words: Polyhydroxybutyrate, Mutagenesis, Halophile.

The accumulation of non-degradable materials waste has significantly increased across the globe due to an exponential growth of human population. The synthetic plastics cause deleterious effects to wild life and pose threat to environment and other serene habitats[1]. The production of biodegradable polymers from renewable resources is the need of the hour, in the face of these ecological facts. There has been considerable interest in the development and production of biodegradable plastics in response to many challenges associated with plastic waste and its adverse effects in the environment. These have led to the research and development on sustainable, compatible, and biodegradable plastic materials, the Bioplastics. Polyhydroxyalkanoate is one such biodegradable microbial polymer which is accumulated in bacteria as intracellular storage granules in the presence of excess carbon sources and limited nitrogen source[2]. The most important consequences of using bioplastics are that they can be produced from renewable sources and they are environment friendly. These so called biodegradable polymers

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or bioplastics were first isolated as lipid-like intracellular storage granules from *Bacillus megaterium*[3]. An efficient bacterial strain is a prerequisite to achieve a cost effective PHA production and is the main focus of interest for many researchers and investigators. Under unfavourable conditions, using acetyl-CoA as an initial material, microorganisms produce polymers and then 3-ketothiolase condenses 2 acetyl-CoAs to acetoacetyl-CoA. Acetoacetyl-CoA is reduced by Acetoacetyl reductase to (R)-3-hydroxybutyryl-CoA and PHA synthase polymerizes (R)-3-hydroxybutyryl-CoA to PHB[4]. Polyhydroxybutyrate (PHB) was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne[5]. The nature of PHB is stiff, highly crystalline, and relatively brittle thermoplastic. It is water insoluble and relatively resistant to hydrolytic degradation. Therefore more efforts are needed to be devoted for making this process PHB is nontoxic, biodegradable and biocompatible and hence is suitable for many medical applications[6].

The present study focuses on the mutagenesis strategies (UV and Acridine orange mutant) to improve and maximise the production of PHB in halophilic strains. In this study, PHB accumulating bacteria were isolated from soil and water samples of Salt Lake Natron, a salt lake situated in Arusha District of northern Tanzania (on the Kenyan border). Further, the bacteria were identified and characterized using morphological, biochemical and molecular techniques and fermented in a suitable media for poly-hydroxybutyrate production.

MATERIALS AND METHODS

Collection of samples

Water and soil samples were collected in small plastic bottles and pouches respectively, from Lake Natron, a salt lake situated in Arusha District of northern Tanzania (on the Kenyan border) at 36 °E longitude and 2° 30' S latitude, in the eastern branch of the East African Rift. It measures a maximum of 57 km long and 22 km wide. The lake is fed by the Southern Ewaso Ng'iro River and also by mineral-rich hot springs. It varies in width depending on its water level, which changes due to high levels of evaporation, leaving behind a mixture of salts and minerals called natron. The surrounding country is dry and receives irregular seasonal rainfall. The lake falls within the Lake Natron Basin Wetlands of International Importance Ramsar Site. Temperatures in the lake can reach 60 °C (140 °F), and depending on rainfall, the alkalinity can reach a pH of 9 to 10.5 (almost as alkaline as ammonia) (Lake Natron, 2014)[7].

Culture and Isolation of Halophiles

Soil samples were diluted (1g/9ml) and the water samples were cultured and grown on petri-plates containing Halobacterium Medium (5g yeast extract, 5g casamino acids, 1g Na-glutamate, 2g KCl, 3g Na_3-citrate, 20g $MgSO_4.7H_2O$, 200g NaCl, 36mg $FeCl_2.4H_2O$, 36mg $MnCl_2.4H_2O$, 1000mL distilled water, pH was adjusted to 8.0)[8]. Cultures were incubated at 37°C for 14-20 days. Colonies of distinct characteristics were observed and further isolation of pure cultures was performed by picking single colony from the plates.

Screening for PHB production

Sudan black stain (Sudan black B powder 0.3 g, 70% ethyl alcohol 100 mL) was used to identify microbial intracellular lipid. Microscopic observations shows lipid inclusion granules are stained blue-black or blue grey, while the bacterial cytoplasm is stained light pink by safranine counter stainning[9]. A loopful of broth culture of PHB producing halophiles was taken and spread on a clean, grease-free slide, covering an area about 10 mm x 30 mm. The smear was allowed to dry in the air and fixed by passing it horizontally through a small Bunsen flame 2–3 times. A few drops of Sudan black solution was placed on the fixed preparation and allowed for 5-10 minutes. The slide was immersed in xylene until it is completely decolourized (about 10 seconds) and allowed to dry. It was further counter-stain with safranine solution for 10-30 seconds. The slide was gently rinsed with running water and allowed to dry again. After the slide was completely dried a drop of immersion oil was added directly and was examined with an oil immersion lens (100X)[10, 11].

Mutant Isolation

Improvement of the microbial strains by inducing Mutations

Random mutagenesis was performed on the isolated halophile strain by both Physical and chemical mutagens. Mutations were induced for improvement of microbial strains for increasing the

production of PHB. Physical mutagen such as UV radiations and Chemical mutagen such as Acridine orange was used in this experimental work.

Inducing mutation by Physical mutagen

Ultraviolet radiations at 254 nm range were used as physical mutagen for inducing mutations in microbial cultures for improvement of strains for PHB. Basically, physical mutagens causes a random mutation in the genome level of the isolates by inducing frameshift mutation, which further leads to deletion, duplication and insertion of nucleotide bases at the DNA genomic level . Physical method was carried out by exposing three glass test tubes each containing 10ml broth culture to UV light (254nm) at different time intervals; 1min., 5min., and 10min respectively[12, 13, 14]. The tubes were then incubated in dark box at 37°C for 1.5hrs. 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 the halophilic media and incubate at 37°C for 14-20 days. Total viable count was measured by Colony Counter[12].

Inducing mutation by Chemical Mutagen

Acridine orange (75¼g/ml) was used as chemical mutagen for inducing mutations for improvement of PHB production of microbial strains. The chemical mutagen Acridine has been studied earlier by many scientists and it was observed that the Acridine exposure induced various dimers formation in the nucleotide bases of bacterial DNA. Different concentrations of Acridine orange solution; 10µl, 50µl, and 100µl were mixed with 10ml broth culture in three different test tubes respectively. The tubes were then incubated in dark box at 37°C for 1.5hrs. After incubation serial dilutions upto 10^{-1}, 10^{-2},10^{-3} were prepared and 0.1 ml of culture was taken and spread over NA media. Incubate for 14-20 days hours at 37°C for further isolation of mutants. Total viable count of colonies was measured by Colony counter[15].

Growth curve determination

A batch culture was prepared by inoculating 10ml pure culture of the isolated strain in 100ml halophilic broth medium in a 250ml conical flask and incubated for 14-20 days at 37°C and 120rpm. Every day, an aliquot of the culture was taken for optical density (OD) determination at 600nm[16].

PHB Extraction

After 12 days of incubation with increased carbon source (dextrose) at 37°C and pH 8.0 in a 2L laboratory scale fermenter at stationary growth phase 10mL of the broth culture was taken and centrifuged at 8000rpm for 15min. The supernatant was discarded and the pellet was treated with 10mL sodium hypochlorite and the mixture was shaken and incubated for 2hours at 30°C. After incubation, the mixture was centrifuged for 15min at 5000rpm and then respectively washed with distilled water, acetone and methanol. After washing the pellet was dissolved in 5mL boiling chloroform and was evaporated by pouring the solution in sterile petriplate at room temperature. After evaporation, the powder is collected for further analysis[17].

TLC

The extracted powdered sample (60mg) was dissolved in 60ul hexane and 4ul of the sample was loaded on the TLC plate containing a solidified silica gel (10g/20ml). It was allowed to run in a solvent system consisting of ethyl acetate and benzene (1:1) for 40mins[17]. For staining, iodine solution was vapourized in a beaker (Borosilicate) at 80-100°C and the TLC plate was placed in the beaker containing the vapourized iodine solution for 5-10 minutes. After then green-black colour was observed indicating the presence of PHB[17]. The R_f (retardation factor) was determined[18,19].

Genomic Characterization of Bacterial isolate

The culture sample was sent to Yaazh Xenomics, Neel Plaza new panvel Navi Mumbai India for the 16s rRNA sequencing of unknown PHB producing strain. Briefly, genomic DNA from the bacterial colony was isolated and the first 500 bp of 16S rDNA were PCR amplified with universal bacterial primers and sequenced. The unknown bacterium was identified using GenBank database[20].

Analysis and Characterization

The extracted powdered sample was characterized by Shimadzu FTIR (Model no. 8400S) spectrometer and Bruker AVANCE III 500 MHz (AV 500) multi nuclei solution NMR Spectrometer (^{1}H), Dimethyl sulfoxide (DMSO) as an internal standard, has been used to determine the structure of PHB. Samples were prepared for analysis by dissolving solvent-cast film segments in deuterated chloroform [1% (w/v)] as a solvent, via mixing with mild heating.

RESULTS AND DISCUSSION

Sample collection and sites

Samples were collected at different sites of Lake Natron.

Colony observation

Different halophile colonies were observed after culture and subculture in halophile medium. Table 1 shows the characteristics of the initial isolated strains.

Strain1 was taken and used throughout the study because of its high viability and faster growth as well as having large intracellular inclusion bodies.

Sudan Black B stainning

The Sudan Black B staining in the analysis show intracellular lipid inclusion inside the bacterial cell wall, which were stained black whereas the bacterial cytoplasm was stained pink (Figure 2).

Mutagenesis

Physical Mutagenesis (UV radiation at 235 nm)

The physical mutagenesis i.e. UV-radiation exposure at different intervals are shown in the table. The table shows that UV- light exposure at 235 nm causes adverse effect on the growth of bacterial isolate strain 1. UV at different time exposures i.e. 3, 5 and 10 minutes effect on the growth of viable colonies. Least number of colonies was observed at 5 min and no viable colonies were observed at 10 min. Thus, UV exposure confirmed that UV mutagenesis affects the viability of bacterial isolates (Table 2).

Chemical Mutagenesis (Acridine Orange)

The Acridine exposure to the halophilic bacteria isolate is shown in the table. The table shows that exposure of chemical mutagen caused adverse affect on the viability of bacterial isolate. As the concentration of Acridine orange increases, the viability of the colonies were nil. Only one colony is observed at 10µl/10ml and no viable colonies were observed at 50 and 100µl/10ml (Table 3).

The growth curve of strain 1

The graph shows the growth curve of halophilic bacterial strain in the Halobacterium medium. The analysis of the growth curve shows an increase in the optical density of bacteria from 1st to 13th day, when inoculated in broth medium, which was spectrophotometrically analysed at 600 nm wavelength (Figure 3).

PHB Extraction

The powdered sample was extracted by sodium hypochlorite method and further quantified in order to known the amount of PHB extracted from the strain 1 before and after conducting mutagenesis experiments. The tables show that the Dry cell weight of PHB in wild strain 1 is 20%. The conduction of Mutagenesis both by physical and chemical agents are shown as 29.63% (DCW powder) induced by UV-light mutagen and 28% (DCW powder) induced by Acridine orange respectively. The results show that the mutations leads to certain changes in the genomic level of

Table 1. Characterization of isolated strains

Characteristics	Strain 1	Strain 2	Strain 3
Morphology	Spiral	Spiral	Rod
Pigmentation	Cream	Orange	Cream
Gram stainning	+	+	-
Catalase	+	+	-

Table 2. Physical mutagen (UV-exposure) at different time intervals

UV-light Exposure time (min)/10ml	Growth	No. of colonies
3	+	7
5	+	2
10	-	0

Table 3. Chemical mutagen (Acridine orange exposure) at different concentrations

Acridine Concentration (µl/10ml)	Growth	No. of colonies
10	+	1
50	-	0
100	-	0

Table 4. Dry cell weight (DCW) of extracted powdered sample of wild, UV mutant and Acridine Orange mutant

Strain 1 (100ml)	PHB yield (%)
Wild type	20
UV Mutant	29.63
Acridine Orange Mutant	28

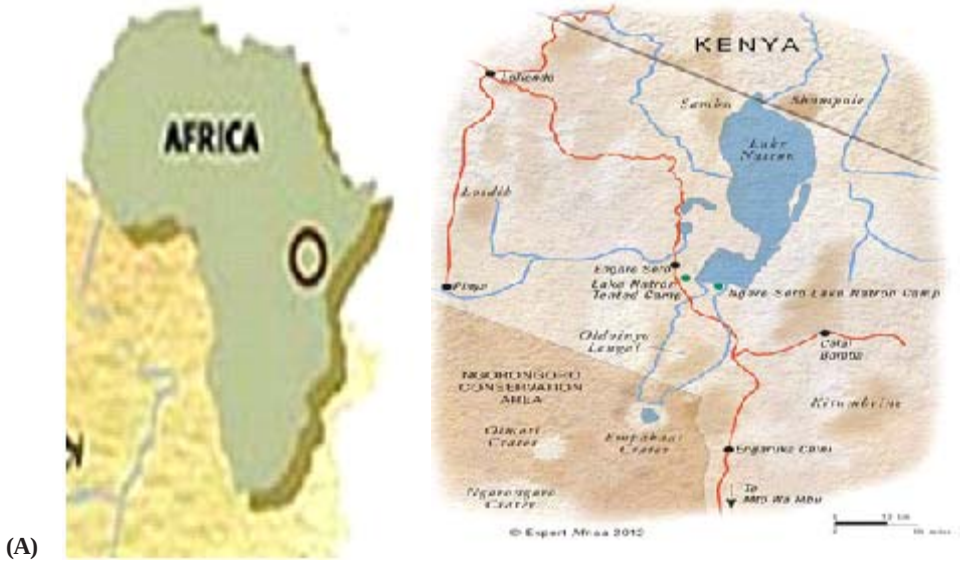

Fig. 1. (A) map of Africa showing location of Lake Natron, (B) magnified location of Lake Natron

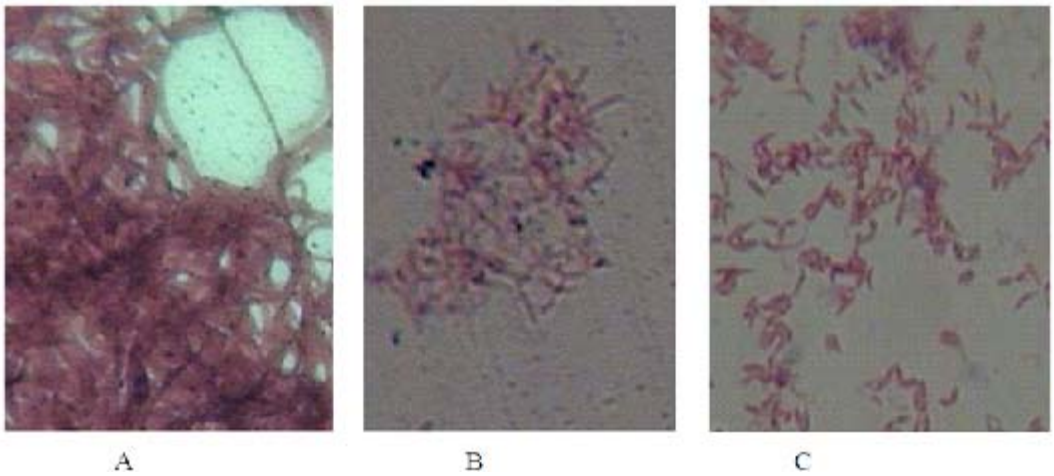

Fig. 2. PHB inclusion bodies A, B and C for strains 1, 2 and 3

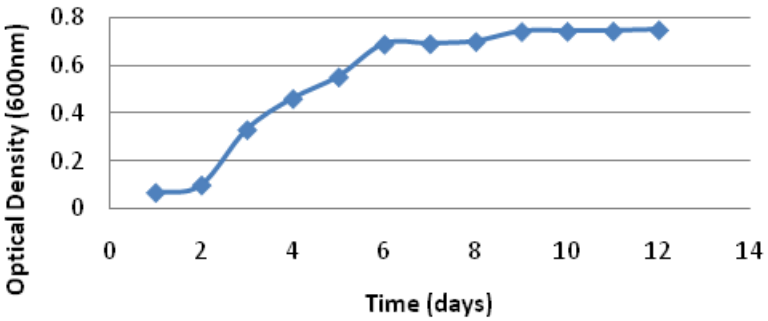

Fig. 3. The growth curve of strain 1 at 600 nm

bacterial isolate. It may also possible that random mutagenesis affects the Pha CAB operon which is responsible for the biosynthetic pathway of PHB production, which leads to an increase in the PHB production of mutant strain 1 (Table 4).

TLC

The thin layer chromatography is an initial confirmatory test to analyse the compound. After running the sample on silica plate, it was stained with iodine vapour. Green-black spot was observed, which indicates that the compound was PHB. The R_f (retention factor) value was measured to be 0.866. Retention factor is defined as the ratio of the distance travelled by the compound to the distance travelled by the solvent (Figure 4).

FTIR analysis

The standard PHB (sigma-aldrich) was analyzed by FTIR in order to known the functional groups present in the chemical structure of PHB at molecular level, taken as standard reference. The characteristic peaks at 1226.77 cm^{-1}, 1454.38 cm^{-1} and 1726.35 cm^{-1} corresponds to the C-O, C-H and C =O shows the functional groups present in the structure of pure PHB (Figure 5).

Analysis of PHB before mutagenesis

FTIR spectrum of extracted sample in Fig 5 revealed the characteristic peaks at 1074.89 cm^{-1} and 1456.1 cm^{-1} which correspond to the C–O and C-H stretch respectively. An additional peak found at 3477.77 cm^{-1} did not belong to PHB and was believed to be caused by the presence of water traces in KBr used for preparing the samples [Figure 6 (A)].

Analysis of PHB after mutagenesis

Similarly, FTIR spectrum of extracted sample revealed the characteristic peak at 1284.7 cm^{-1}, 1460.16 cm^{-1} and 1728.28 cm^{-1} which corresponds to the C-O, C-H and C =O stretch respectively. Number of additional peaks shows the presence of water traces in KBr used for the sample preparation [Figure 6 (B)].

The extracted samples before and after mutagenesis were analyzed by FTIR, which shows similar peaks, when compared with standard PHB. The analysis revealed that extracted samples

Fig. 4. TLC analysis of extracted PHB

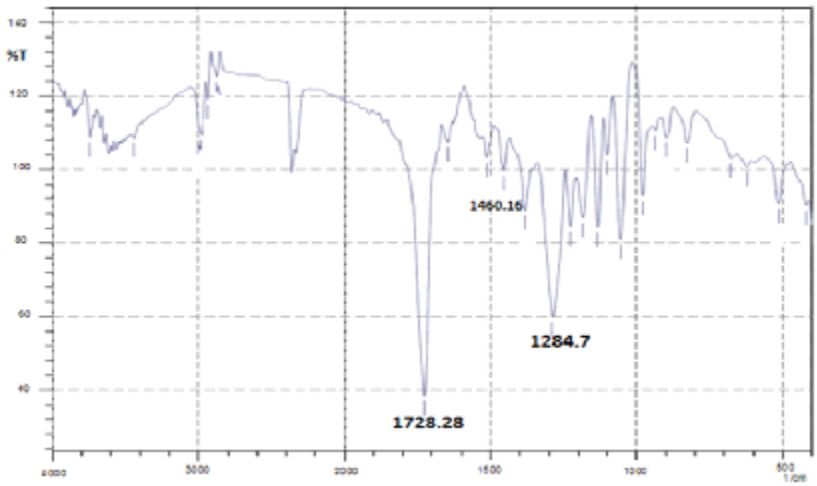

Fig. 5. FT-IR analysis of Standard PHB (Sigma-Aldrich)

belonging to the member of family Poly-hydroxyalkoanate i.e. Poly- hydroxybutyrate.

Characterization of Extracted Polymer

The ^{1}H NMR spectra obtained from extracted PHB from the isolated strain M1 was compared with the standard PHB (Sigma-Aldrich Chemicals, USA). Both spectra were found to match perfectly with each other. The peaks observed in the spectra coincide, corresponding to the different types of carbon atoms in the PHB structure. The spectrum shows a doublet at 0.82ppm which is attributed to the methyl group coupled to one proton where as in mutant strain, it was observed at 0.79. The doublet of quadruplet at 1.20ppm and 2.49ppm is attributed to the methylene group adjacent to an asymmetric carbon atom bearing a single atom and in mutant strain methylene group was observed at 1.22 and 2.49ppm. The multiplet at 5.1ppm is characteristic of methine group in PHB (Sigma Aldrich), whereas

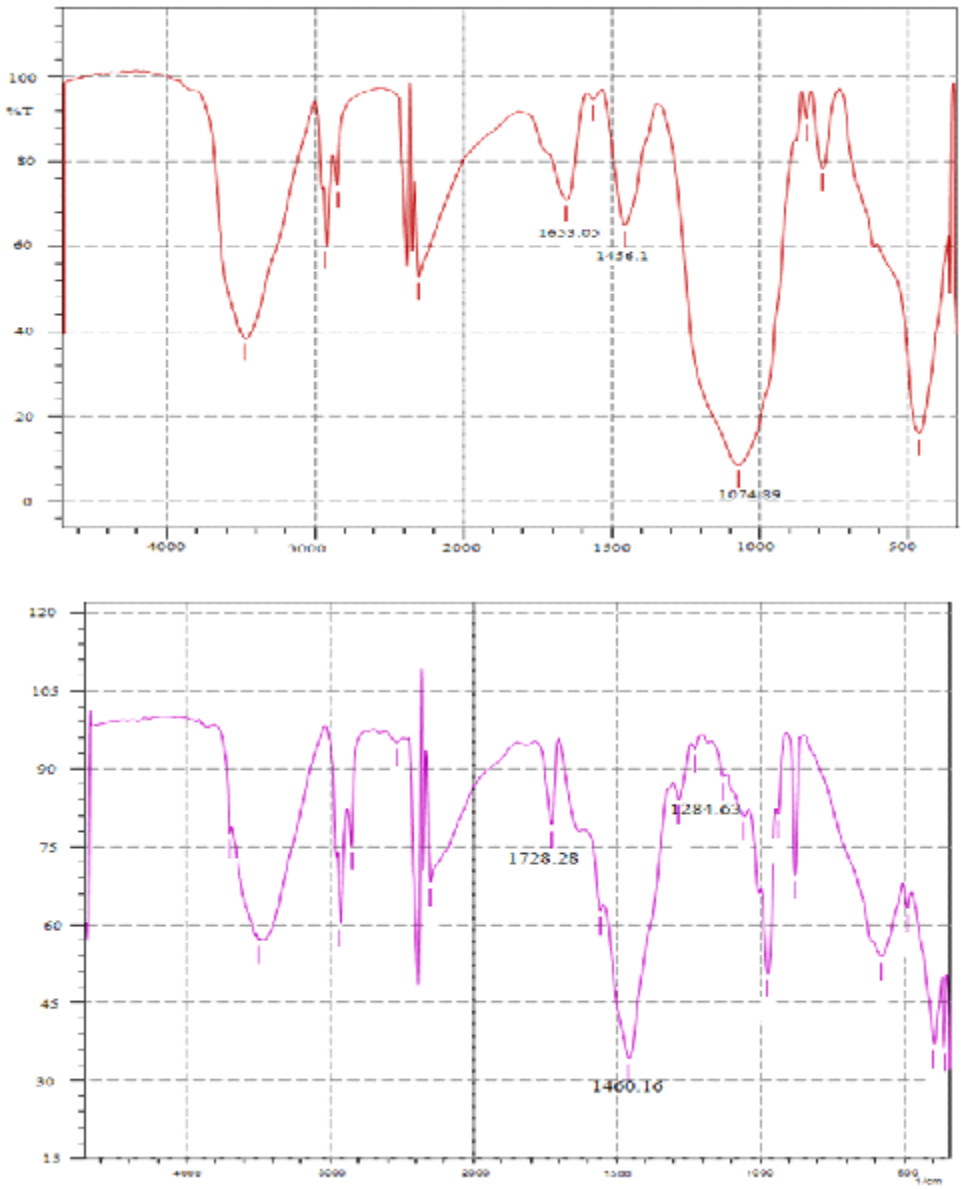

Fig. 6. (A). FT-IR analysis of extracted sample before Mutagenesis (UV mutant), (B) FT-IR analysis of extracted sample before Mutagenesis (Acridine Orange mutant)

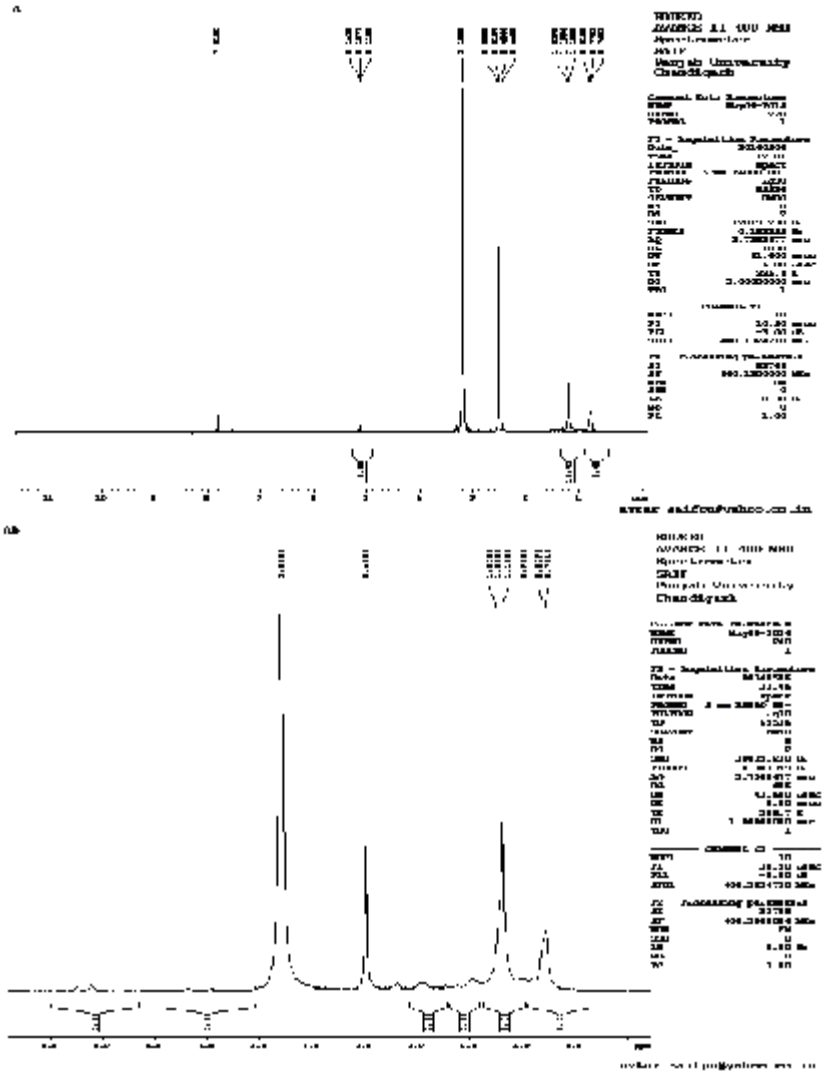

Fig. 7. Nuclear magnetic resonance spectrometry (H-NMR) analysis of PHB produced in strain1. A Bruker Avance II 400 NMR Spectrometer was used. (A) NMR of PHB produced from wild type strain1 processed at 400.13 MHz, (B) NMR of PHB produced from the mutant strain 1 processed at 400.1300 MHz

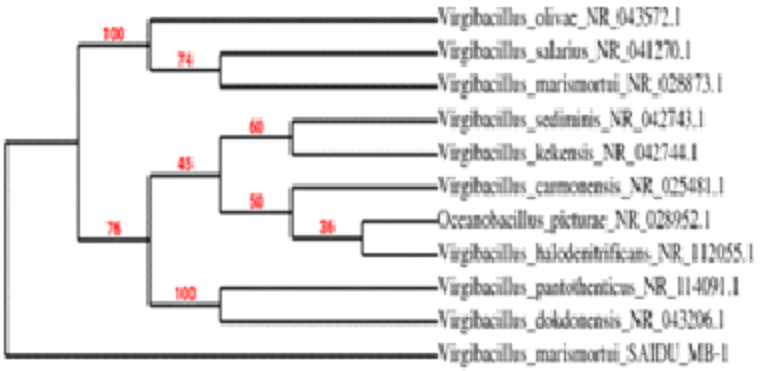

Fig. 8. Phylogenetic tree of strain1

in extracted PHB from bacteria, it was analysed at 3.29ppm. Two other signals are observed, a broad one at 1.23ppm was observed in PHB (Sigma Aldrich), whereas 1.25ppm was observed in extracted PHB which is due to water and another one at 7.81ppm (Sigma Aldrich) and 8.1 attributed to the solvent used i.e. chloroform (Figure 7).

16s rRNA Sequencing

The halophilic strain was identified as *Virgibacillus marismortui* SAIDU MB1 (Acession no. KJ939620) (Figure 8).

DISCUSSION

Lake Natron is a salt lake and is probably the world's most caustic body of water. Due to the saline nature of the lake, many salt loving bacteria are thriving there and are characterised as halophiles (extremophiles). The red accessory photosynthesizing pigment present in the cyanobacteria (helps in photosynthesis) gives red colour to the lake. Three strains of halophiles with distinct characteristics were isolated as shown in table1 and only one strain was used throughout this study because of its high viability and faster growth as well as has high PHB inclusion bodies inside the cell. One of the strains has a characteristic of producing orange colour pigment.

Physical (UV light) and chemical (Acridine orange) mutagens were highly effective. The lethality of Acridine orange mutant was tremendous compared with the UV light mutant. The organism was able to survive after 3 and 5 minutes exposure time under the UV light (table2). However, Acridine orange confers lethal effect on the organism at 50µl concentration per 10ml broth culture (table3).

The PHB yield varies considerably between the wild type strain and the mutant. The PHB yield increased from 20% (Wild type strain) to 28% (Acridine orange mutant) and 29.63% (UV light mutant). The highest yield was observed in the physical (UV light) mutagen. This indicated that for a significant increase in PHB yield UV light could be a suitable mutagen. Moreover, Pal *et al*., (2009) reported that among the UV-mutants screening for high PHB granules were analyzed as compared to the parental strain.

Both physical and chemical mutagens have been used to improve industrial strains. Hikmet *et al*., (2003), have reported a significant increase in the PHB yield of *B. megaterium* Y6, *B. subtilis* K8, and *B. firmus* G2 through mutation. Also, Dave *et al*., (1996) reported DCW of 70% PHB in optimum culture conditions for *Bacillus* spp. IPCB-403. Findlay and White (1983) reported the presence of PHB in *B. megaterium* chromatographically[22]. Chen *et al*., (1991) studied D (-)-3-hydroxyalkanoate in 11 different *Bacillus* spp. and observed that 50% of DCW accounted for PHB in the bacteria. Many of the *Alcaligenes eutrophus* strains used for PHB production on commercial scale have DCW of approximately 96% PHB[23].

There has been an increasing research on PHB as a substitute for non-biodegradable petroleum-based plastics. Most research efforts are carried out in this field for the isolation of PHB-producing microorganisms from different sources and improving the PHB production ability of microorganisms via less expensive substrates and genetic modification for high-yield biopolymer production.

CONCLUSION

This study yielded a putative UV mutant strain with increased PHB production from 0.05 g/100ml to 0.08 g/100ml, and increased DCW PHB from 20% to 29.63% as compared to the wild type strain. The ^{1}H NMR results also shows similar peaks as compared with PHB procured from Sigma-Aldrich. Further much more analysis could be carried out at the protein and gene level for molecular characterization of such mutant strain, which may shed more light upon the strategies that could be used for targeted strain improvement. Growth and media optimization research with the mutant will not be left behind. The mutation may help in the development of potential industrial strains high PHB production, so that it could be commercialised. Finally, the results of this work may be used for the development of innovative fermentation strategies for the production of PHB and might aid to define novel targets for the genetic manipulations of PHB producing bacteria.

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