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ORIGINAL ARTICLE **OPEN ACCESS**

Isolation, Screening and Characterization of New Strains with Optimization Studies to Augment Bacterial PHB Production

Mukesh R. Jangra and KS Nehra
Biotechnology Department, Govt PG College, Hisar-125004, India
Email-jangra.mukesh02@gmail.com

ABSTRACT
Bacterial Polyhydroxybutyrate (PHB) is a green plastic that is completely biodegradable and leaves behind no scum. In the present study, high PHB producing strains were isolated from soil and sewage samples. Three strains named M6, M12 and M14 were found to accumulate PHB granules with Nile Blue A staining. These three strains were morphologically and biochemically characterized for primary identification. Growth profiles were studied for all the three strains using PHB production medium by assessing the level of PHB polymer synthesis after 24h, 48h, 72h, 96h and 108h of inoculation at 37°C. Among the three strains, M12 showed highest PHB production in the form of Crotonic acid (O.D. 0.895) after 48h. Further, In Gel Lysis Electrophoresis of selected bacterial strains was performed for the plasmid profiling for characterization and identification of bacterial strains. The present study provided useful data about optimization of conditions for PHB production that can be utilized in industrial production of PHB, a fast emerging substitute of petroleum based plastic. Characterization and confirmation of PHB was done using FTIR and NMR.

Keywords: *Bioplastics, Biodegradable, Nile Blue A, Polyhydroxybutyrate,*

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are aliphatic biopolymers that fully comply with requirements like *biobased, biodegradable, compostable or biocompatible* compounds to meet the criteria to convert these into *Green plastics.* Bioplastics are plastic like biopolymers which have their origin from biological sources such as microorganisms. Polyhydroxyalkanoates are the polyster of hydroxyacids which are naturally synthesized. Among all bio-based plastics, they are exclusive by being entirely produced and degraded by microorganisms under natural environment. Bacteria accumulating PHA in the presence of excess carbon source is similar to how humans accumulated fat deposits in their body. Poly-3-hydroxybutyric acid is the most common microbial polyhydroxyalkanoates. They have molecular weights ranging from 50,000 to 1,000,000 Da (1). The non-degradable plastic reportedly accumulates at rate of 25 million tons per year (2). Polyhydroxybutyrate accumulated as energy reserve material in many micro-organisms and can accumulate upto 70% of their dry weight of PHB. These microorganisms could produce the polymer in environment of nitrogen and phosphorous limitation. Several investigations suggested that microorganisms like *Alcaligens eutrophus* (3) and *Alcaligens latus, Actinobacillus, Azotobacter, Agrobacterium, Rhodobacter, Sphaerotilius* and *Rhizobium* have focused on converting organic waste to bacterial PHA and these microorganisms are able to form a material with a variety of characterstics. Gram negative bacteria have been found to be the better producers of PHB because the outer membrane of these bacteria (*Cupriavidas necator, Alcaligenes latus*) contains LPS endotoxins that may induce a strong immunogenic reaction which limits its biomedical application. On the other hand, Gram positive bacteria lack LPS and they secrete protein at higher concentration and the potential use of cheaper raw materials made it a better source of PHA. Many bacteria including those in the soil are capable of PHB production and breakdown (4). A series of enzymes: synthetases or depolymerases are implied in the biosynthesis and biodegradation of poly-β-hydroxybutyrates and also of other polyhydroxyalkanoates (5). In their metabolism, three biosynthetic enzymes worked to convert acetyl-coenzyme-A (acetyl-CoA), into PHB. The first reaction is the condensation of two acetyl-CoA molecules into acetoacetyl-CoA by beta-ketoacyl CoA thiolase (encoded by *phbA*). The second reaction consists of the reduction of acetoacetyl-CoA to (R)-3- hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl- CoA dehydrogenase (encoded by *phbB*).

Finally, the (R)-3- hydroxybutyryl- CoA monomers are polymerized into PHB by P(3HB) polymerase, encoded by *phbC* (6).

The major objective of this study was: (i) to isolate novel PHB producer (ii) to assess the ability of *Bacillus* and *Pseudomonas* strains to accumulate PHB under different conditions such as incubation time, carbon source and nitrogen source. In addition, the most important feature is that *Bacillus* and *Pseudomonas* spp are among those few organisms, which can produce homopolymer and co-polymer of PHA from pure substrates as well as agribioproducts (7). (iii) This study was aimed at revealing PHB inclusions from *Bacillus* and *Pseudomonas* species isolated from soil with the objective to determine the carbon and nitrogen source that will give the highest yield of PHB.

MATERIALS AND METHODS

Sample collection and isolation of pure cultures

Sewage and soil samples were collected from Hisar, Haryana and stored in sterile bottles. One gram of sample was dispensed in 10ml of sterile distilled water. This was mixed vigorously and 1ml from this mixture was taken and added to another tube containing 9ml sterile distilled water to get a dilution of 10^{-1}. This serial dilution was repeated to get dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. For the isolation of PHB producing bacteria, 0.1ml of each dilution was plated on to a nutrient rich medium (Minimal Medium) by spread plate method. The plates were incubated at 37^0 C for 96 hours. Colonies with different characteristic features were maintained as pure cultures on nutrient agar slants and stored at 4°C.

Screening of bacterial strains for PHB production

For screening the PHB producing bacterial isolates, Nile blue A staining method was used. A stock of Nile blue A stain (10 mg/ml) was prepared in DMSO and filter (11 µm) sterilized. This Nile blue A stain was added to the autoclaved medium at a final concentration of 25 µg/ml and were screened under UV light after 3 or 4 days of incubation The PHB producing strain gave a orange/yellowish color fluorescence. The extent of fluorescence indicates quantity of intracellular PHB.

Optimization of cultural parameters for maximum PHB production

The factors (carbon and nitrogen sources) which are affecting PHB production of the selected bacterial isolates were optimized. For the optimization of different carbon source on PHB production, the production medium in each plate was consisted of minimal salt medium, potassium nitrate as a common nitrogen source and a different carbon source like fructose, glucose, maltose, sorbitol, chitin, peptone, CMC, dextrose, glycerol, mannitol, starch, galactose and molasses (3-5g/L). Whereas, for the optimization of different nitrogen source on PHB production, the production medium in each plate was consisted of minimal salt medium, glucose as a common carbon source and a different nitrogen source like ammonium chloride, ammonium nitrate, ammonium acetate, ammonium sulphate, cotton cake, potassium nitrate, sodium azide, mustard cake, yeast extract and urea (3-5 g/L). The plates were incubated under stationary conditions at 37^oC. The extent of fluorescence shown by bacterial isolates using different carbon/nitrogen source indicates the relative amount of PHB production.

Morphological and biochemical characterization of PHB producing bacterial isolates

PHB producing strains were identified and characterized morphologically and biochemically according to the Bergey's Manual of Determinative Bacteriology (8). For morphological characterization, the bacterial cultures were grown on nutrient agar media and stained using gram staining method. Whereas, for biochemical characterization, different tests like MR-VP test, catalase test, urease test, mannitol utilization test and triple sugar iron test (9) were carried out.

Growth curve studies for PHB producing bacterial isolates

The selected bacterial isolates were cultivated in a 250 ml erlenmeyer flask using 50 ml of minimal salt medium (Beringer et al., 1978) containing 2g/l glucose (carbon source), 0.5g/l KNO_3 (nitrogen source), 0.5g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$, 0.1g/l NaCl and 0.06g/l $CaCl_2.2\ H_2O$ under stationary conditions at 37^0C for 24 hrs . Initial pH (7.0) of the medium was kept constant. Further, to study PHB production growth curve of the bacterial isolates, one litre of the above media was taken in a 2000 ml flask and the flask was inoculated with 50 ml of the inoculum. The flask was kept under stationary conditions for 96 hrs at 37^oC. Samples were withdrawn at regular intervals of 24h and analyzed for PHB recovery and PHB weight. Cell pellet was collected by centrifuging bacterial culture broth at 10,000 rpm and the cell pellet was boiled in 10 ml sodium hypochlorite (4%). For PHB recovery, after cooling, 20 ml of chloroform was added and the sample was kept at 65^0C. At this temperature, chloroform evaporated and dissolved PHB was recovered. 50 µl of dissolved PHB was added to 2 ml sulphuric acid and incubated for 24h at 65ºC. The amount of dissolved PHB was analyzed spectrophotometrically at 235 nm. The bacterial isolates which gave higher PHB recovery and weight were selected for further studies.

Preparation of standard curve

Standard curve of PHB was prepared following the method of Slepecky & Law (11). Two gram of pure PHB was dissolved in 10 ml of concentrated H_2SO_4 and heated for 10 min. to convert PHB into crotonic acid, which gave 200 mg/ml of crotonic acid. From the above stock, working standard solutions were prepared by diluting it to obtain different concentrations ranging between 10 mg/ml to 150 mg/ml. Absorbance of all the dilutions was read at 235nm against a concentrated H_2SO_4 blank on UV-VIS spectrophotometer, standard graph was plotted between concentration of crotonic acid and O.D. at 235 nm. By referring to the standard curve, the quantity of PHB produced was determined. The assays were done in triplicates.

Characterization of PHB:

Fourier Transform Infrared Spectroscopy (FTIR) and NMR were done to characterize PHB. The presence of different functional groups in PHB was checked by FTIR. Extracted PHB (2 mg) were dissolved in 500 µl of chloroform. After evaporation of chloroform, PHB polymer film was subjected to FTIR. For Nuclear Magnetic Resonance (NMR) spectroscopy, the polymer was suspended in spectrochem grade deuterochloroform ($CDCl_3$). The 1H NMR spectra of sample was obtained at 300 MHz IIT, Delhi. The chemical shift scale was in parts per million (ppm).

RESULT AND DISCUSSION

Investigations were conducted to isolate, characterize bacteria capable of polyhydroxybutyrate (PHB) production and to find out the best possible physiological conditions for better PHB production.

Sample collection and isolation of bacterial strains

PHB producing bacteria are reportedly ubiquitous, very diverse in their habitat and properties. From five diverse locations (Table 1) such as oil, spilled soil, sewage water and decomposed waste, seventy bacterial strains were isolated using spread plate and streak plate methods. Maximum no. of cultures was collected from sewage samples obtained from Hisar, India.

Table 1: Sources of bacterial isolates

Sr. No	Sources	No. of bacteria isolated	No. of Nile Blue A positive isolates
1	Hisar farm soil	11	3
2	Hisar sewage water	13	4
3	Oil spilled soil	18	3
4	Sewage sludge	13	2
5	Hisar canal water	15	3

Screening of bacterial strains for PHB Production

The extent of fluorescence is proportional to the intracellular amount of PHB present. Out of seventy bacterial isolates, only fifteen **(M1 to M15)** were found to produce PHB. These fifteen isolates were further screened on different carbon and nitrogen sources. Ostle and Holt (12) reported that Poly-β-hydroxybutyrate granules exhibited a strong orange fluorescence when stained with Nile blue A, a specific dye for PHB granules. It was further confirmed by the study of Bhuwal et al., (13), where they isolated 120 isolates from different soil samples and out of these, 62 isolates showed positive results with Nile blue A staining, . Nile Blue A is more specific stain for PHA than a more rapid and sensitive, viable colony method. This dye directly included in carbon-rich nutrient agar medium, and growth of the cells occurred in the presence of the dye. The PHA accumulating colonies, after Nile blue A staining, showed bright orange fluorescence on irradiation with UV light and their fluorescence intensity increased with the increase in PHA content of the bacterial cells. The isolates which showed bright orange fluorescence on irradiation with UV light after Nile blue A staining were selected as PHA accumulators and the results obtained were similar to Cortes et al. (14).

PHB production on diverse sugars as carbon sources

Analysis of relative PHB production as indicated by the extent of fluorescence suggested that bacterial isolates M6, M12 and M14 were the highest producers almost on all media containing different carbon sources. Majority of the isolates grew better when glucose was used as the sole carbon source (Table 2). The next promising carbon source was fructose which was utilized by most of isolates. In this screening, +1 and +2 score has given to the culture for low florescence whereas +3 and +4 were high florescence giving culture. Glucose and fructose, being monosaccharides were readily utilizes by bacteria, hence their growth and subsequent production of PHB was higher. In case of sucrose, Maltose and chitin, complexity

of the carbon increased and hence PHA yields were low. Similar conclusion was made by Chandrashekharaiah et al. (15). However, for the PHB production by *Rhizobium* bacteria, sucrose was found to be better source of carbon.

Table 2: Screening for relative PHB production on different Minimal medium containing Nile blue A stain with different carbon source

S.No.	Isolates	Glucose	Fructose	Galactose	Peptone	Tryptone	Maltose	Sucrose	Chitin	Starch	Sorbitol	mannitol	glycerol
1	M1	2+	2+	1+	1+	2+	2+	1+	2+	2+	2+	2+	2+
2	M2	2+	2+	-	-	2+	1+	2+	1+	2+	2+	2+	
3	M3	2+	2+	-	-	-	-	-	-	-	-	-	-
4	M4	1+	1+	-	-	-	-	-	-	-	-	-	-
5	M5	2+	2+	-	-	2+	1+	2+	2+	2+	2+	2+	2+
6	**M6**	**3+**	**2+**	**1+**	**1+**	**3+**	**1+**	**2+**	**2+**	**2+**	**2+**	2+	2+
7	M7	2+	3+	-	1+	2+	1+	1+	1+	-	-	-	-
8	M8	1+	2+	-	-	1+	-	-	2+	2+	1+	2+	2+
9	M9	2+	+1	-	-	1+	-	-	1+	2+	1+	2+	2+
10	M10	1+	1+	-	-	2+	-	2+	1+	2+	1+	2+	2+
11	M11	1+	1+	-	-	2+	1+	2+	1+	1+	1+	2+	2+
12	**M12**	**3+**	**1+**	**-**	**3+**	**3+**	**2+**	**3+**	**2+**	**2+**	**2+**	2+	3+
13	M13	1+	-	-	-	-	-	-	-	-	-	-	-
14	**M14**	**3+**	**1+**	**1+**	**3+**	**3+**	**3+**	**4+**	**1+**	**2+**	**3+**	3+	3+
15	M15	2+	-	-	-	-	-	-	-	-	-	-	-

Note- +1 and +2 score has been given to the culture for low florescence whereas +3 and +4 were high florescence giving culture on the basis of visual observation.

Aarthi and Ramana (16) also suggested that *Bacillus mycoides* DFC1 had shown preference for carbon sources like glucose, maltose and wheat starch. Yüksekdağ et al. (17) observed the highest level of PHB accumulation in the medium with glucose as carbon source in *B. subtilis* 25 (19.51%), *B. megaterium* 12 (19.49%). According to Hori et al. (18), in *B. megaterium* PHB content in the cell was reached a maximum level after growth with glucose. Muralidharan and Radha (19), revealed that the PHB yield was the

highest when glucose was used as carbon source with 55mg PHB/g cell weight, Glucose was the preferred carbon source and was taken for other studies. The positive effect of glucose on PHB production could be attributed due to the increased supply of the reduced cofactor, i.e. NADPH which in turn leads to the inhibition of TCA cycle enzymes.

Contribution of diverse nitrogen sources on the extent of PHB production

The nitrogen sources are the secondary energy sources for the organisms which play an important role in the growth of the organism and the production of PHB. On the basis of growth pattern on different nitrogen sources, strains M6, M12 and M14 showed maximum florescence and utilized almost all nitrogen sources. Majority of the isolates grew better when potassium nitrate was used as the sole nitrogen source (Table 3). The results were in agreement with those of Kannahi and Rajalakshmi (20), who reported highest PHB productivity in potassium nitrate (0.052 g/L) compared to other nitrogen sources such as ammonium sulfate (0.039 g/L) and glycine. Elsayed et el. (21), also designed modified culture medium for PHB production containing 0.7% glucose and 100 mg/L potassium nitrate as a carbon and nitrogen sources, respectively and PHB production was increased from 24 to 42% per dry weight. Potassium nitrate gave much more satisfactory result among the selected nitrogenous source as suggested by Aswini et al. (22). According to Lakshmi, et al., (23), by using potassium nitrate as nitrogen sources, *Rhizobium* produced maximum level of PHB upto (8.6 %). Apart from it, other studies showed that maximum PHB production (0.052 g/l) was observed in bacterial isolates *Pseudomonas aeruginosa* and *Rhizobium* spp when potassium nitrate was used as the nitrogen source (24) and these results were in agreement with study of Jangra and Sikka, 2015 (25).

Table 3: Screening for relative PHB production on different Minimal medium containing Nile blue A stain with different nitrogen source

S.No.	Isolates	KNO_3	NH_4SO_4	NH_4NO_3	NH_4CL_2	$CH_3COO\ NH_4$	Yeast Extract
1	M1	2+	-	1+	1+	-	-
2	M2	2+	-	-	1+	-	-
3	M3	2+	-	1+	2+	-	2+
4	M4	-	1+	-	2+	1+	1+
5	M5	2+	-	-	1+	-	-
6	**M6**	**3+**	**2+**	**2+**	**2+**	**3+**	**3+**
7	M7	2+	-	-	-	-	-
8	M8	1+	1+	-	1+	1+	1+
9	M9	2+	-	3+	-	-	-
10	M10	1+	-	-	-	-	-
11	M11	1+	-	-	-	-	-
12	**M12**	**3+**	**3+**	**3+**	**3+**	**3+**	**3+**
13	M13	1+	1+	3+	2+	1+	3+
14	**M14**	**3+**	**3+**	**3+**	**3+**	**3+**	**3+**
15	M15	2+	-	1+	2+	-	-

PHB extraction and quantitative estimation

On the basis of screening on different carbon and nitrogen sources, M6, M12 and M14 were further selected for PHB estimation. The amount of PHB produced by each selected isolate is shown in Table 4. The amount of cells in the pellet collected from one litre culture medium was quantitated which corresponded to the growth rate of bacterial culture. Crotonic acid assay was the most prominent and feasible quantification technique for the production of PHB. Crotonic acid measurements were done to analyze actual amount of pure PHB produced by the selected strains. The principle behind this assay is that the PHB crystals undergo degradation on treatment with sulphuric acid (26). The extracted PHB was converted to crotonic acid by adding 98% sulphuric acid and heated to 60°C for 1 hour. Crotonic acid shows maximum absorption at 235nm.The absorbance of the solution as measured at 235nm in a UV Spectrophotometer. Fig. 1 shows the standard curve of PHB. The standard curve was used for estimation of PHB yield of the bacterial isolates (27, 28, 29). Among the three selected bacterial isolates, M14 was found to be producing the maximum cell pellet and maximum O.D., whereas strain M12 produced minimum cell pellet on dry cell pellet basis and producing least crotonic acid (least O.D. value). M6 was the second cell pellet producer and the crotonic acid production was also maximum after M14 (Table 4).

Table 4: Comparison of PHB production using selected bacterial isolates (96h incubation)

Sr. No.	Strains	Cell pellet wt.(gm/l)	PHB expressed as Crotonic acid (OD 235)	PHB wt.(g/l)
1.	M6	0.77±0.011	0.790±0.005	0.69±0.011
2.	M12	0.69±0.012	0.614±0.001	0.54±0.012
3.	M14	2.57±0.012	0.914±0.003	0.81±0.012

Note: Values are represented as Mean ± Standard Deviation

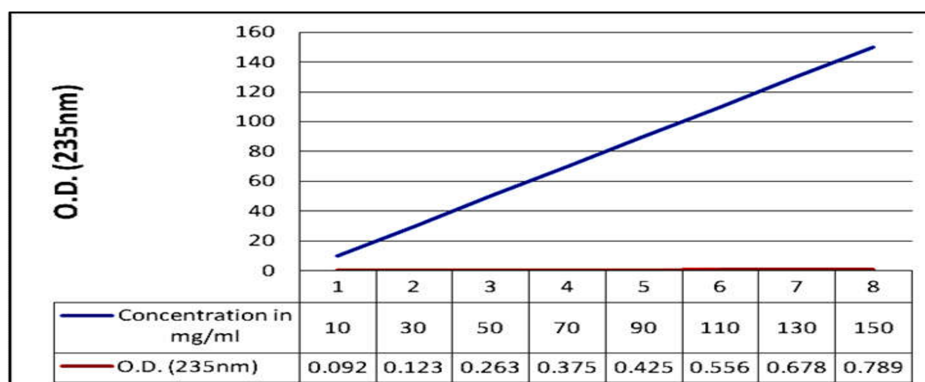


Fig 1. Standard graph of PHB production

Characterization and evaluation of selected bacterial strains

Microbiological, cultural and biochemical tests were performed for characterization of selected PHB producing bacterial strains, for their tentative generic identification (Table 5).

Table 5: Characterization of selected bacterial isolates for growth properties

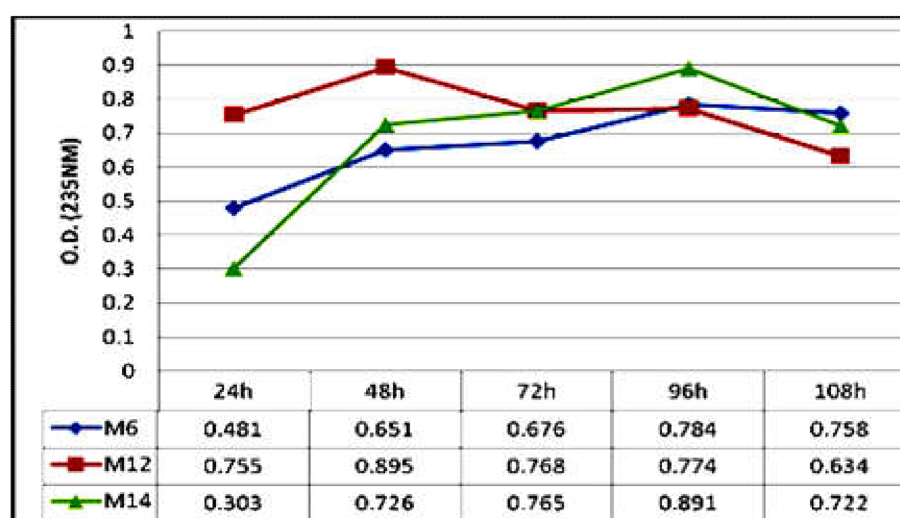
1.Morphological characterization	**M6**	**M12**	**M14**
Shape	Round	Round	Round
Gram stain	-ve	+ve	-ve
Spore stain	-ve	+ve	-ve
Congo red stain	-ve	-ve	-ve
2.Cultural characteristics			
Colony color	Orange	Creamish white	Orange
Colony morphology	Round	Gummy round	Round
Temperature	Optimum	Optimum	Optimum
Growth on agar plate	Abundant	Abundant	Abundant
Growth on Kings'B medium	+ve	-ve	+ve
3.Biochemical characterization			
Indole production	-ve	-ve	-ve
Methyl-Red Test	-ve	+ve	-ve
Voges-Proskauer test	+ve	-ve	+ve
Citrate utilization test	-ve	+ve	-ve
Urease	-ve	-ve	-ve
Gelatin	-ve	-ve	-ve
Catalase	+ve	+ve	+ve
Triple Sugar Iron Test	-ve	+ve	-ve
Mannitol utilization test	-ve	-ve	-ve
Proteolytic activity test	-ve	-ve	-ve
Cellulose hydrolysis	-ve	-ve	-ve
Phosphate solubilisation	-ve	-ve	-ve
Tentative Genera designated	*Pseudomonas* sp.	*Bacillus* sp.	*Pseudomonas* sp.

Cultural, morphological and biochemical characterization revealed that M6 and M14 isolates were *Pseudomonas* and M12 was *Bacillus.* An analysis of the capacities of different microbes to produce PHAs established that certain Gram-negative bacteria species belonging to *Alcaligenes, Ralstonia* and *Pseudomonas* lead this group. *Pseudomonas* can normally synthesize mcl-PHA on various aliphatic alkenes or fatty acids, agricultural and oily wastes (30, 31). *Pseudomonas* sp. can, however, simultaneously produce scl-mcl PHAs (32, 33, 34, 35). Poly 3- (hydroxyalkanoates) production ability of *Psudomonas*

aeruginosa was reported from oily substrate and fatty acids [36, 37]. *Pseudomonas fluorescens* has previously been experimentally shown to produce PHB and its genomic sequence indicates the presence of the Pha A/B/C/Z genes involved in PHB metabolism [38]. It has several bioremediation applications to degrade pollutants [39]. Apart from it, certain Gram-positive bacteria reported to produce PHB and copolymers include *Bacillus, Clostridium, Corynebacterium, Nocardia, Rhodococcus, Streptomyces* and *Staphylococcus* [40] *B. subtilis* is already known for production of valuable metabolites, bioremediation and generation of bioenergy. Among the *Bacillus spp*. reported to be PHA producers, the PHA yields vary from 11 to 69% (w/w of DCW – upto 70 g/L): *B. amyloliquefaciens* DSM7, *B. laterosporus, B. licheniformis*, *B. macerans*, *B. cereus*, *B. circulans*, *Bacillus* sp. INT005 and *B. cereus* UW85 could produce PHA with a wide range of compositions varying from PHB depending on substrates. Various *Bacillus* spp. have been shown by different researchers to synthesize copolymers when co-fed with various substrates.

PHB production kinetics

As per the result shown in Fig. 2, it was observed that strain M12 gave maximum PHB production within 48h and, strains M6 and M14 reached to maximum PHB production within 96h. After 96h, there was a marked decrease for crotonic acid formation. Similar results were shown by Najeh *et al*. [41]. Finally, strain M12 was selected because it took less time (48h) and gave maximum PHB production.



	24h	48h	72h	96h	108h
M6	0.481	0.651	0.676	0.784	0.758
M12	0.755	0.895	0.768	0.774	0.634
M14	0.303	0.726	0.765	0.891	0.722

Fig 2: Kinetics of PHB production in three strains expressed as crotonic acid (O.D. 235 NM)

Time-course analysis (Fig. 2) indicated that PHB was a growth associated product and its accumulation significantly increased when the culture reached stationary phase (about 48-96 hrs). The maximum O.D. values were achieved at 48 h in case of strain M12. After 52 hrs, a slight decrease in the level PHB content. This indicated that the presence of an intracellular PHB depolymerase. Similar studies were done by Gowda et al. (42) in *B. thuringiensis* IAM12077 which showed that PHB yield increased from 0.533 g/L at 0h to 4g/L by 48h and later gradually declined. Concentration of PHB decreased significantly after 60 hrs in M12 strain due to nutrient depletion and cells start consumption of PHB as a carbon source. The lower total dry cell weight of the bacteria corresponded with high amount of PHA production within 48 hrs cultivation these was similar *Ralstonia eutropha* (recent name *Cupriavidus eutropha*), which accumulated PHB at the stationary phase (43). M12 produced maximum PHB at the 48th hour of incubation followed by a similar decline. M12 strain achieved maximum level of PHB within 48h under similar growth conditions, making it a more industrially viable strain relating to faster productivity as compared to M6 and M14. In case of M6 and M14 strain, maximum values were achieved at 96 and 72 hrs.

Characterization of PHB

FTIR Analysis

The Fourier Transform Infra Red (FTIR) spectrum of the PHB sample isolated from strain M12 revealed 6 major peaks at 2921, 2955, 1637, 1619, 1378 and 1458 (fig.3).

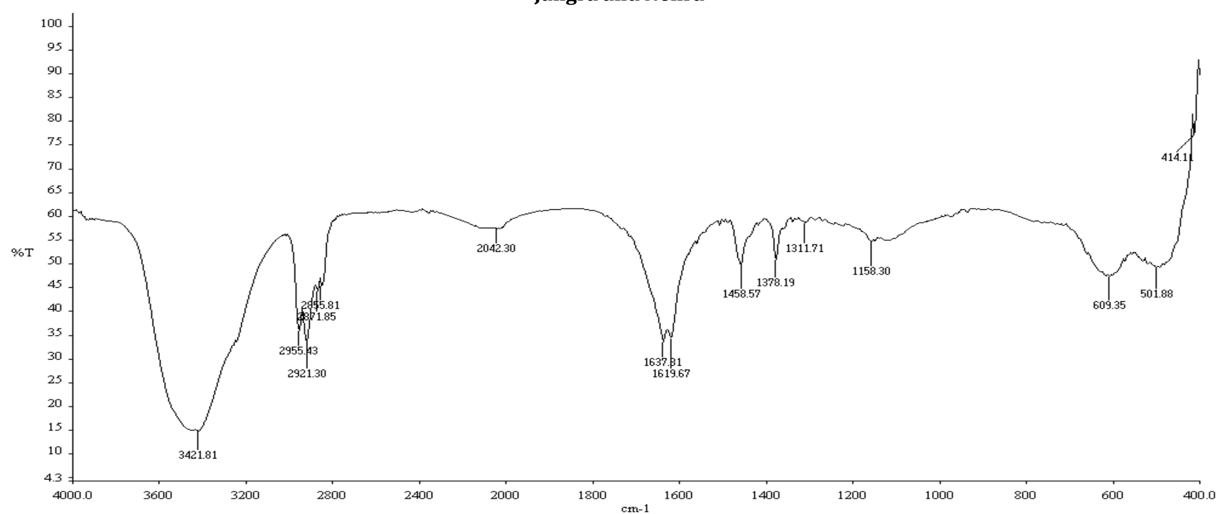


Fig. 3: FTIR spectra of PHB extracted from strain M12

The predominant peaks at 2921-2960 and 1378-1496 which represents the methine groups, followed by a peak at 1637 which shows the presence of –C=O. The presence of these marked peaks demonstrated the presence of PHA. On the basis of data obtained in the present work it was concluded that the *Bacillus sp* is capable of producing PHB and that can be used for industrial purpose for the manufacture of biodegradable plastics. Similar results were shown by Muthazhagan and Thangaraj [44] and Rajendran *et al.* [29]. All the absorption due to the PHB moiety appeared in the spectrum and other spectra at different positions, indicating the possible accumulation of various PHA in cell further analysis was required to investigate whether there were other hydroxyl alkanoates present. This led to investigate the H-NMR spectroscopic data of the compound.

NMR analysis

1H NMR shows the results from PHB synthesized by strain M12. The peaks observed in the spectra (fig. 4) correspond to the different types of carbon atoms present in the PHB structure. ^{1}H NMR spectra showed peaks at 0.843-0.855 ppm and 1.260 which represent methyl groups. These results were identical to those reported by Ashby et al. (45).

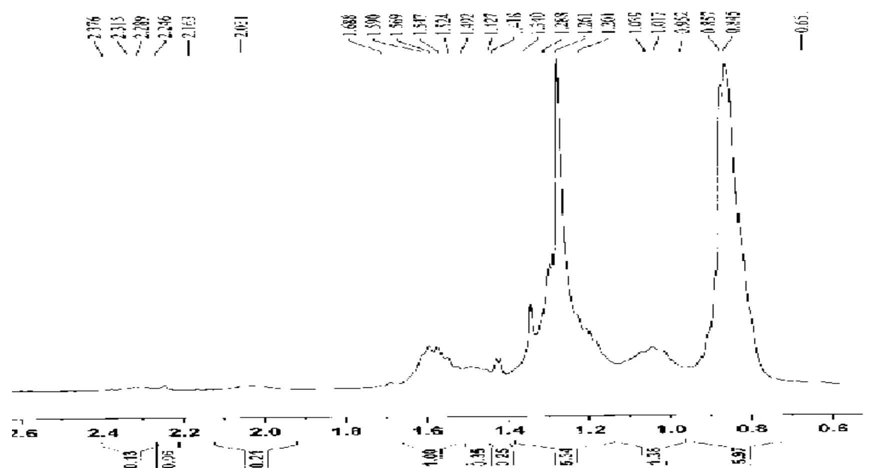


Fig 4: ^{1}H NMR spectra of PHB produced from strain M12

From these results, it can be concluded that Bacillus sp. M12 cells produce PHA exclusively in the form of PHB. These observations were supported by the investigation of Bhuwal et al. (13).

CONCLUSION

The problem of plastic pollution is now really a muddle for mankind. There is no part of the world which is safe and sound from its impact. In the present era of globalization some strategy must be there to combat such a big environmental problem. Making science to the leap and forgetting the other side of coin lead to such conditions. In the present study three novel strains M6, M12 and M14 were isolated and characterized. These were found to be *bacillus* and *pseudomonas* species and these are able to produce

biodegradable plastic. This approach is safe and ecofriendly. *Bacillus* species (M12) isolated from the soil samples can be employed in the industrial production of PHA due to the less time it is taken to produce PHB. Additionally, these bacterial strains are able to degrade the polymer intracellularly by using the enzyme PHA depolymerase. Currently, these bacterial strains are further studied to increase the productivity of PHB by observing the effect of various carbon and nitrogen supplementation including agribyproducts to make this process cost effective.

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