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Bioanalytical Evaluation Of PHA Co-Polymer Produced By *Massilia Spp* Isolated From Rhizosphere Soil

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Abstract: Polyhydroxyalkanotes are naturally occurring biodegradable polymers. The aim of the present study was to optimize and characterize PHA extracted from a rhizosphere soil isolate. 16S rRNA sequencing revealed that isolate belongs to the genus *Massilia.* Confocal laser scanning microscopy revealed the presence of bright orange colored fluorescent granules when grown under nitrogen limiting conditions and stained with Nile red dye. Present isolate was known to produce highest amount of PHA when supplemented with maltose as a carbon source and NH_4HPO_4 as a nitrogen source. After optimization, isolate produced 2.0g/l of PHA. FTIR spectrum of PHA revealed the presence of C=O, -OH and C-O bonding. T_d of extracted polymer was found to be at 281^{0}C. NMR and GC-MS spectrum revealed that it is a co-polymer of 3-hydroxybutyric acid (3HB) and 9, 19-dihydroxy octadecanoic acid (HOD).

Keywords: Polyhydroxyalkanoate, co-polymer, *Massilia spp,* Confocal laser scanning microscope, 3- Hydroxybutyric acid, Octadecanoic acid.

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1. INTRODUCTION

The genus *Massilia* belongs to the family Oxalobacteraceae and was first described by La Scola *et al.* (1998).[1] Thereafter, many species of *Massilia* were isolated from environmental samples which include species from air (*Massilia aerilata, Massilia norwichensis*), freshwater (*Massilia aurea*) and soil (*Massilia humi, Massilia armeniaca, Massilia flava*). Although they are ubiquitous, little is known about their ecological importance such as degradation of aromatic compounds, PHA production.[1-7] Polyhydroxyalkanoates (PHAs) are polyesters produced as intracellular granules by a large number of microorganisms. PHAs accumulate during unbalanced growth conditions, usually characterized by an excess supply of carbon and/or limitation of one or more essential nutrients, such as nitrogen, phosphorus or oxygen.[8] PHAs can be divided into three major groups based on the number of carbons in their monomer units, such as short-chain length (SCL), medium-chain length (MCL) and long-chain length (LCL) consisting of hydroxyacids with 3 to 5, 6 to 14 or more than 14 carbon atoms respectively. PHA composition and physicochemical properties are influenced by diverse factors such as the nature of substrates provided (alkanes, alkenes, alcohols, fatty acids, carbohydrates) and the specific active metabolic pathways present. [8,9] Majority of bacteria either synthesize SCL or MCL PHAs. Polyhydroxybutyrate (PHB) is the commonest member of the PHA family. It belongs to the Short Chain Length (SCL) PHA. PHB is synthesized by a wide variety of bacteria (*Ralstonia eutropha, Azotobacter beijerinckii, Bacillus megaterium*) to name a few.[10] MCL-PHA is synthesized by only *Pseudomonas spp* (*P.oleovorans, P.putida, P.aeruginosa*).[11] Few bacterial species such as *Aeromonas caviae, Rhodococcus ruber, Nocardia corallina, Bacillus megaterium and* some of *Pseudomonas* (*P.fluorescens, P.marginalis, P.mendocina and Pseudomonas sp 61-3*) are known to accumulate the co-polymer of SCL-MCL PHAs with a maximum skeleton upto C_{14}. Sludge isolate *Pseudomonas aeruginosa MTCC 7925* is the only isolate reported so far to produce a novel SCL-LCL co-polymer with a carbon skeleton greater than C_{14} i.e. with C_{16} and C_{18} with SCL components of C_4 and C_5.[9] The present study deals with isolation and identification of PHA producing bacteria from rhizosphere soil. One factor at time (OFAT) approach was used in order to optimize carbon and nitrogen source. Further characterization of PHA was carried out by using different bioanalytical techniques.

2. MATERIALS AND METHODS

All medium components were purchased from HI-MEDIA and Chemicals were purchased from LOBA- CHEMIE. Medium used: Carrot infusion agar, Quarter strength Nutrient broth 34(b), Mineral salt medium (MSM).[12]

2.1 Isolation, identification and screening of PHA producer

Moist rhizosphere soil was placed on moistened filter paper. Washed rice was sprinkled on soil and incubated at room temperature (RT) for 48 hrs. Rice grains which were partially covered with a purple, membranous growth were then placed on carrot infusion agar and incubated at RT for 24-48 hrs. The purple pigmented bacterial isolate was identified by 16S rRNA sequencing. Purple pigmented soil isolate was grown in quarter strength Nutrient broth 34(b) under shaken conditions (150 rpm) for 18-24 hrs, at 28^{0}C. 50 ml of the Mineral salt medium (MSM) supplemented with 1% Glucose as production medium was inoculated with seed (1%) and shaken (150rpm) at 28^{0}C for 48 hrs. The grown culture was analyzed to check for their PHA production ability primarily by Sudan Black B and secondarily by Nile red staining.

2.2 Nile red staining

100 µl of the stationary phase grown culture (48 hrs) was withdrawn, centrifuged at 8000g, for 10 minutes. Pellet obtained was washed with 100 µl methanol and acetone mixture (1:1), centrifuged at 15000 rpm for 10 minutes at 4^{0}C. 100 µl of Nile Red solution (10mg/ml in acetone) was added to the pellet, incubated in the dark for 10 minutes and centrifuged at 15000 rpm for 10 minutes. Pellet was washed twice with phosphate buffered saline (PBS), re-suspended in phosphate buffered saline and observed under confocal laser scanning microscope.[13]

2.3 Extraction of PHA using sodium hypochlorite digestion method

10 ml of MSM broth grown cells were harvested by centrifugation at 8000 rpm for 15 minutes at the end of the incubation period (48-72 hrs). The pellet obtained after centrifugation was dried at 60^{0}C to remove moisture. PHA extraction from dried pellet was carried out by sodium hypochlorite digestion method. Dried pellet was treated with 10 ml of sodium hypochlorite (4%) at room temperature for 90 minutes and subsequently centrifuged at 8000 rpm for 15 minutes. The pellet obtained was then sequentially washed twice with distilled water and acetone: methanol (1:1). Washed pellet was then dissolved in 10 ml of hot chloroform, filtered through Whatman filter paper no-1 and filtrate was then evaporated to dryness to obtain PHA powder.[14]

2.4 Optimization of cultural parameters for maximum PHA production

Optimization of fermentation medium for enhanced production of metabolites is crucial. Therefore, in order to determine optimum conditions for utilization of carbon and nitrogen source one factor at a time (OFAT) approach was used. Growth conditions such as temperature, pH, incubation time, agitation speed were optimized for the given isolate.[15] The effect of different carbon sources on growth and PHA production was determined by growing the isolate in MSM supplemented with different carbon sources such as glucose, maltose, fructose, sucrose, starch and glycerol (10g/l) and ammonium chloride (NH_4Cl) as a nitrogen source (0.5 g/l). The selected bacterial isolate was then grown in MSM supplemented with best carbon source and different nitrogen sources such as inorganic (NH_4Cl, NH_4HPO_4, NH_4NO_3 and $(NH_4)_2SO_4$) and organic (tryptone, peptone, yeast extract and beef extract) to study the effect of nitrogen source on growth and PHA production.

2.5 Characterization of polymer

2.5.1 FTIR

The purified dried sample was subjected to IR analysis (Bruker, Germany) to identify the functional groups present

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in the polymer. IR spectrum was recorded in the range of 400-4000 cm^{-1}.[10]

2.5.2 TGA

Thermal properties of the polymer were determined using a PERKIN ELMER, USA Thermo gravimetric analyzer. 10–20 mg of purified polymer was placed on an aluminium pan and heated from 20^0C to 500^0C at the rate of 20^0C /min, under purged nitrogen.[16]

2.5.3 C – NMR

NMR spectroscopy was used to identify the purified polymer. For C-NMR analysis, 20 mg of purified polymer was dissolved in deuterated chloroform and spectra were recorded on JNM-ECZ600R/S1 spectrometer at 600 MHZ.[17]

2.5.4 GC-MS

Monomer composition of PHA was determined by using GC-MS after methanolysis of the sample. For methanolysis, 20 mg of polymer sample was dissolved into 2 $cm^3$of chloroform and 2 cm^3 of acidified methanol and placed in polytetrafluoroethylene-lined screw-cap test tube. 0.5mg of benzoic acid per cm^3 of the solvent was added as an internal standard. Mixture was heated at 100^0C for 140 min and rapidly cooled to room temperature and 2 cm^3 of distilled water was added. The solution was vortexed for 1 min and allowed to separate into aqueous and organic phases. The organic phase was removed and subjected to analysis by GC-MS using a flame ionization detector (FID). 1 µL of sample was split injected on to a 30-m EB-5 (5% diphenyl-95% dimethyl polysiloxane) column with a 0.25mm ID (Restek, Bellefonte, PA). The injection port was held at 280^0C and the oven housing column was held at 100^0C for 3 min. The oven temperature was then raised at the rate of 8^0C/min to 280^0C and the column was held for 2 min. Thereafter the oven temperature was raised to 310^0C at the rate of 20^0C/min and held for 10 min to remove any residuals from the column.[10]

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of rhizosphere soil isolate

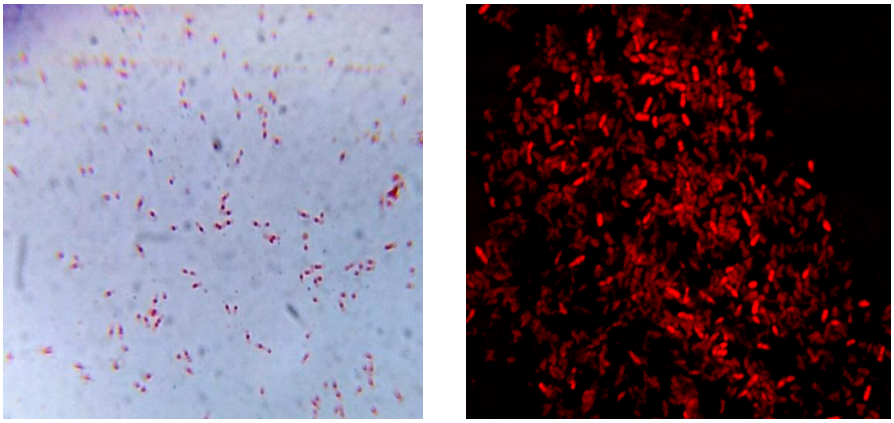
After the incubation at RT for 48 hrs, purple mucoid exudate was observed on rice grains, indicating growth of purple pigmented bacteria (Figure I A).

A B

Fig I. (A) Purple mucoid growth observed on rice grains (48hrs) (B) Growth pattern on Carrot infusion agar (72hrs)

Carrot infusion agar was used for the isolation of purple pigmented bacteria as carrots are a good source of several vitamins, amino acids and minerals. Among all these amino acids, vitamin A and tryptophan are necessary for purple pigment production. After 48-72 hrs of incubation large, dark purple, raised, rough colonies appeared, which were difficult to pick up and unable to be dispersed in saline (Figure IB). 16S rRNA partial sequencing identified the isolate as *Massilia spp* (GenBank Accession: MH730065.1). Members of the genus are aerobic, Gram-negative, motile, short or straight rods with flagella, catalase and oxidase positive.

3.2 *Screening of bacterial isolate for PHA production using Nile red staining*



A B

Fig II. (A) Sudan Black B staining B] Nile red staining of rhizosphere bacterial isolate grown in (MSM) supplemented with 1% glucose after 48 hrs of incubation (B) Confocal laser scanning microscopy of and stained with Nile red stain.

Preliminary screening for the detection of lipid granules was done by Sudan black B staining method. Fig (IIA) shows the presence of black lipid granules inside irregular pink colored cells. This was further confirmed by staining with Nile red stain and visualizing under confocal laser scanning microscope. Nile red, when in lipid rich environment can fluoresce intensely and produce varied colors from deep red to strong gold yellow emission. The presence of bright orange colored granules was observed which almost filled the entire cytoplasm (Figure IIB).

3.3 *Optimization of cultural parameters for maximum PHA production*

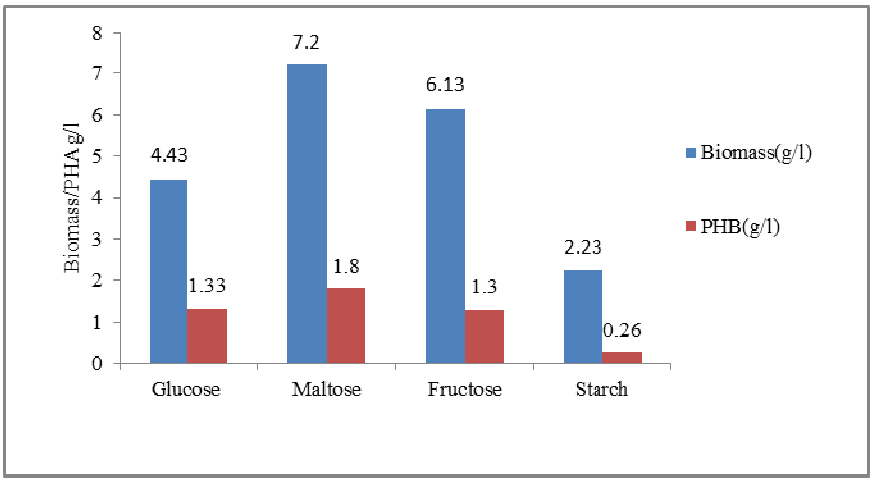


Fig III. Effect of carbon source on growth and PHA production

Among all the carbon sources tested to study their effect on PHA production, maltose was found to be the best carbon source for maximum polymer production (1.8g/l) (Figure III). Lowest PHB production was observed with starch as carbon source (0.26g/l). No growth was observed when supplemented with sucrose and glycerol as a carbon source. Earlier studies revealed use of simple carbohydrates – such as glucose for maximizing production of PHA. Cerrone *et al.* (2011) reported PHA production by several species of *Massilia (M.plicata, M.dura, M.aurea, M. albidiflava, M. brevitalea, M. lutea, M. aerilata)* when grown on ISP2 medium supplemented with either glucose or starch as carbon source .[1] The best yield was reported by *M. lutea* when medium supplemented with starch as carbon source. Bassas-Galia et al.(2011) reported that among all eleven isolates of *Massilia*, isolate 4D6 was found to achieve maximum PHA yields when grown on glycerol containing medium.[8] Present isolate known to utilize maltose as a carbon source for the production of maximum PHA. Both inorganic and organic nitrogen sources supported cell growth as well as PHA production (Figure IV). Highest amount of PHA was produced when supplemented with NH_4HPO_4 (2.0g/l) followed by NH_4Cl (1.6g/l), NH_4NO_3 (1.2g/l), beef extract (1.17g/l), tryptone (1.07g/l), yeast extract (0.93g/l), peptone (1.03g/l), and $(NH_4)_2SO_4$ (0.83g/l). The isolate prefers to utilize inorganic nitrogen sources than organic for maximum PHA production. These results are in good agreement with Aramvash et al. (2015) who observed highest PHA production when NH_4Cl was used as nitrogen source by *Cupriavidus necator*.[18] Lathwal et al. (2015) reported use of $(NH_4)_2SO_4$ as best nitrogen source for maximum PHA production by *Micrococcus spp* isolated from rhizosphere soil.[15]

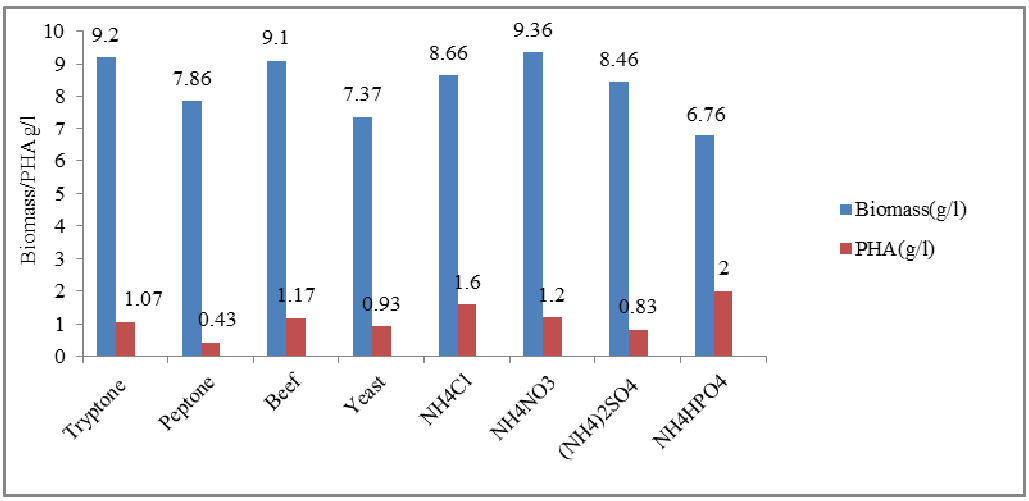


Fig IV. Effect of nitrogen source on growth and PHA production

3.4 Characterization of polymer using different bio-analytical techniques

FTIR spectrum of polymer extracted from *Massilia* confirms the presence of functional groups (Figure V). From the spectrum it was inferred that band near 3436 corresponds to terminal hydroxyl (-OH) group whereas band at 1723 represents ester (C=O) group. C=O group is a diagnostic signal for the presence of PHA. The band at 1455 corresponds to C-H group showing asymmetrical stretching and bending vibrations in CH_3 group. Bands ranging from 1057 to 1279 represent C-O bonding. Similar IR spectra were recorded by Devi et al. (2015) and Kiran et al. (2014) confirming the presence of ester (C=O) linkage in their extracted polymer sample.[16,19] This is further confirmed and identified by TGA, C-NMR and GC-MS.

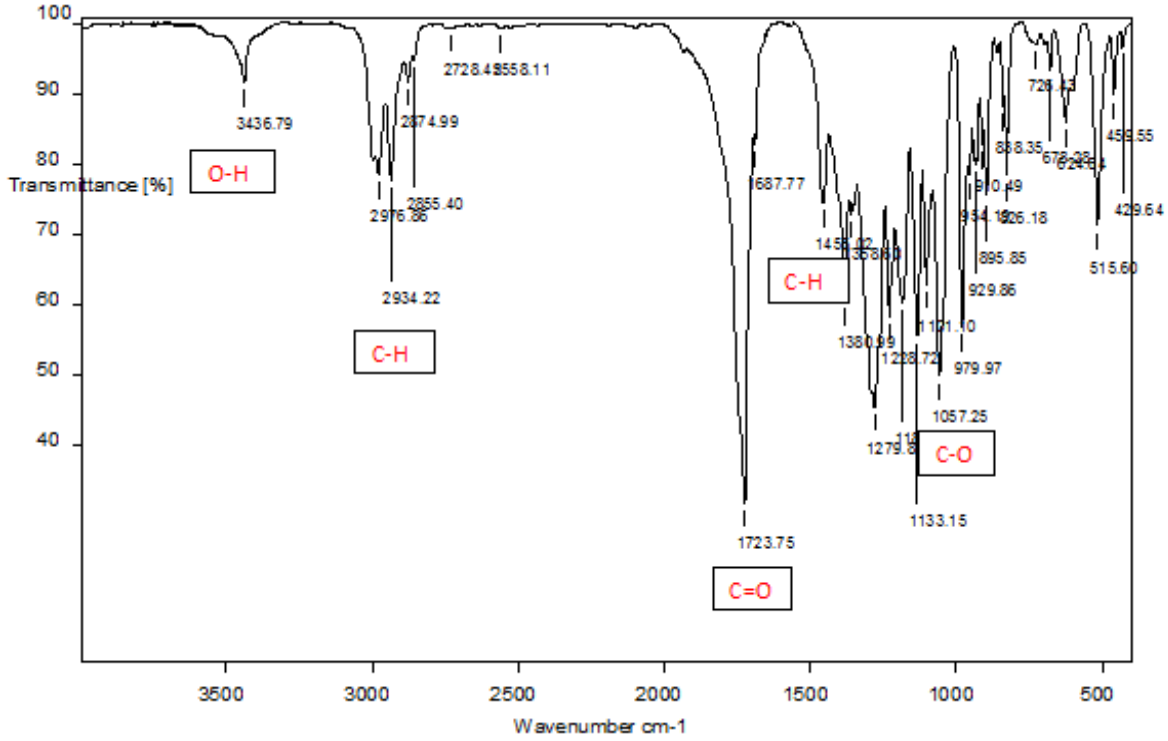


Fig V. FTIR spectrum of polymer extracted from *Massilia spp*

^{13}C-NMR study confirms the presence of polymer containing 3HA (3-hydroxy acid) units of the 3HB (3-hydroxybutyric acid) and 3HOD (hydroxyoctadecanoic acid) (Figure VI). The peak at 19.826 ppm is assignable to $-CH_3$ of 3-hydroxybutyric acid (3HB). The peak at 40.825 ppm corresponds to $-CH_2$ of main chain of 3HB. The peak at 67.676 ppm is assigned to –CH of the main chain of 3-hydroxybutyric acid (3-HB) and 3-hydroxyoctadecanoic acid (3HOD). The resonance from 76.830 to 77.303 ppm is assignable to $CDCl_3$ (solvent). Further peak at 169.135 ppm is due to the carbonyl resonance in 3HB*-3HA. This data indicates, it is a co-polymer of 3-hydroxybutyric acid (3-HB) and hydroxyoctadecanoic acid (HOD). The chemical shift signals of ^{13}C-NMR obtained in this work are quite similar to the spectra reported by Singh et al (2008) where *P. aeruginosa* MTCC 7927 synthesized co-polymer of SCL- 3HB and LCL- 3-hydroxyhexadecanoic acid (3HHD) & 3-hydroxyoctadecanoic acid (3HOD).[20] Although numerous studies reported production of poly(hydroxybutyrate co hydroxyvalerate) i.e SCL copolymer by several bacterial species, no other report has been documented for copolymer production by *Massilia* spp so far.

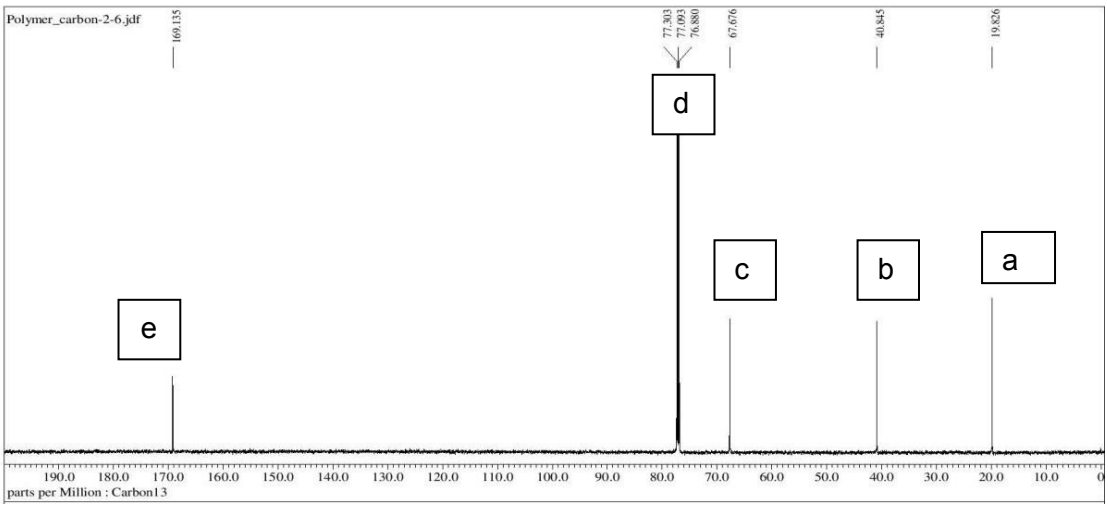


Fig VI. ^{13}C-NMR spectra of polymer extracted from *Massilia spp*: (a) – CH_3 of 3HB, (b) – CH_2 of 3HB, (c) - CH of 3HB + 3HOD, (d) – $CDCl_3$,(e) -CO group of 3HB+ 3HOD

GC-MS chromatogram of methanolyzed PHA sample revealed five major peaks. On the basis of mass spectral library, they were identified as follows: benzoic acid methyl esters (a-reference std) , butanoic acid 4-methoxy methyl ester(b-C:4), 3- Hydroxybutyric acid, 2-(2-t-butoxycarbonyl)-1-methoxy methyl ester (c & d- C:4) and 9,19-dihydroxy octadecanoic acid(e-C:18)(Figure VI). Pappalardo et al. (2014) reported that, when grown on refined glycerol medium, *Pseudomonas mediterranea* 9.1 has shown major peaks of MCL –PHA such as pentanoic acid methyl ester (C5), decanoic acid methyl ester (C10), dodecanoic acid methyl ester (C12) whereas *P. corrugata* A1 showed four relevant peaks out of eight: pentanoic acid ester (C5), decanoic acid ester (C10), dodecanoic acid methyl ester (C12) and hexadecanoic acid methyl ester (C16).[11] Singh et al (2008) determined the composition of polymer synthesized by *P. aeruginosa MTCC 7925* and observed that it is a copolymer of SCL-PHA (3-Hydroxybutyric acid & 3-Hydroxyvaleric acid) and LCL-PHAs of C16 (3-Hydroxyhexadecanoic acid) and C18 (3-Hydroxy octadecanoic acid).[20] PHA of our isolate was compared with the above data. It was observed that 3-HB has side chains and esterification of octadecanoic acid has happened at 9th and 19th position. To date there are no reports of other bacterial species (except for *Pseudomonas sp*) able to synthesize such a long chain hydroxy acid i.e C:18.

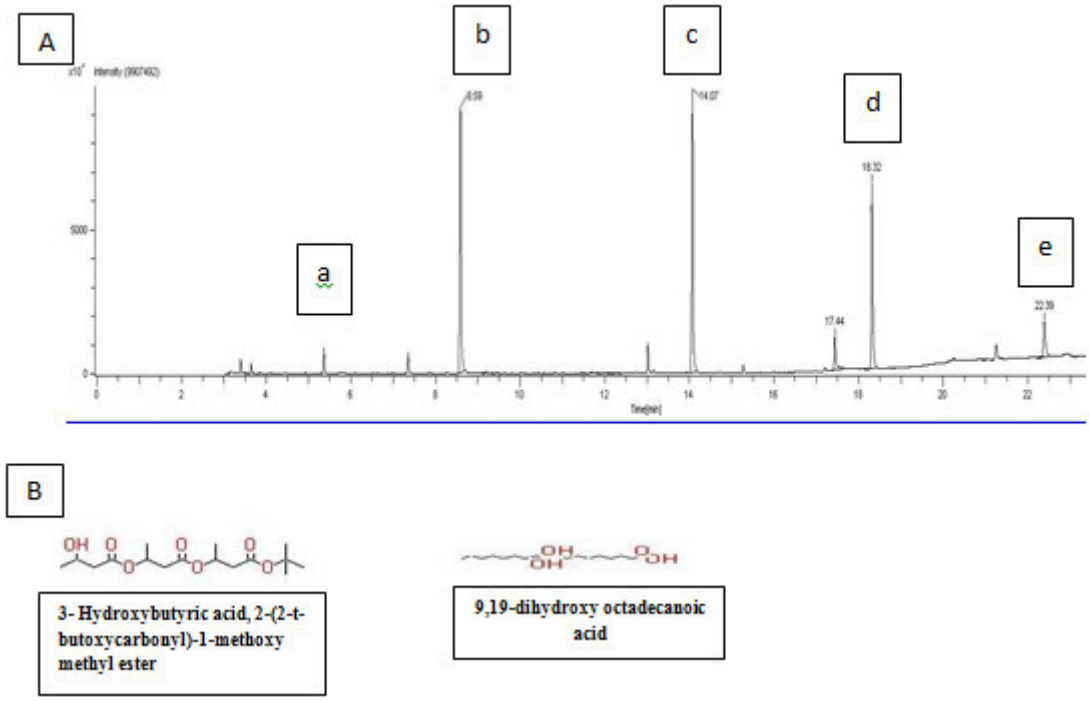


Fig VII. A) GC-MS chromatogram of extracted polymer from *Massilia spp* B) Structure of c and e a)Benzoic acid methyl ester , b) Butanoic acid 4-methoxy methylester, c) & d)3- Hydroxybutyric acid, 2-(2-t-butoxycarbonyl)-1-methoxy methyl ester, e) 9,19-dihydroxy octadecanoic acid

Thermal stability of extracted co-polymer was studied with respect to the temperature at 5% weight loss ($T_{d(5\%)}$) (Figure VIII). The ($T_{d(5\%)}$) of extracted polymer was found to be at 281^{0}C.($T_{d(5\%)}$) for copolymer which was significantly higher than the PHB homopolymer ($T_{d(5\%)}$- 235^{0}C). Enhanced thermal stability of co-polymer P(3HB-co-9,19-HOD) could

be due to steric hindrance in the formation of six member ring produced by longer side chains. Similar results were recorded by Singh *et al* (2013). Bassas-Galia et al. (2011) reported that polymer extracted from *Massilia* strain 4D6 had decomposition temperature (T_d) values between 295–298^{0}C which is slightly higher than our polymer. [8,17]

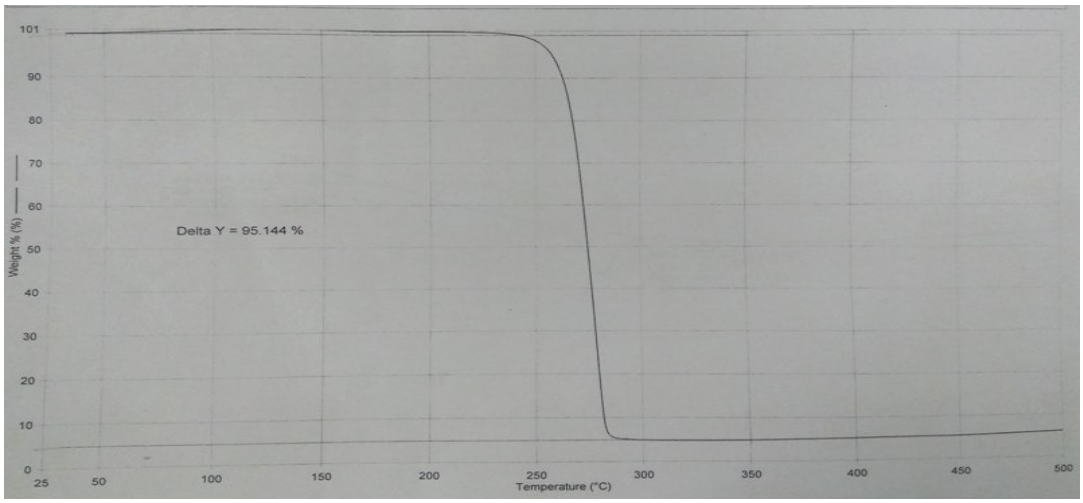


Fig VIII. TGA curve for extracted polymer

4. CONCLUSION

The purple pigmented soil isolate belongs to the genus *Massilia*. When MSM broth was supplemented with maltose as carbon and NH_4HPO_4 as nitrogen source, isolate has ability to produce 2.0gm/l of PHA after 48 hrs of incubation. FTIR, TGA thermogram, C-NMR, GC-MS confirmed the presence of a co-polymer of SCL-PHA(3-HB:C4) and LCL-PHA(9,19HOD:C18). The organism seems to be a potential candidate for PHA production on a large scale.

5. ACKNOWLEDGEMENTS

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6. AUTHORS CONTRIBUTION STATEMENT

Ms.Priyanka Naik conducted research and gathered data with regard to this work. Dr. Vaijayanti Ranade and Ms. Priyanka Naik together analyzed data and necessary inputs were given towards the designing of the manuscript. All authors discussed the methodology and results contributed to the final manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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