

Exploring the Characterization of biodegradable plastic “polyhydroxybutrate” (PHB) from *Bacillus siamensis* PHB 01 (MW440618) isolated from termite mound soil of Western Ghats of Coimbatore, Tamil Nadu.

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Research Article

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Abstract

The present study was aimed to explore the characterization of polyhydroxy butrate extracted from the bacterial strain under optimized conditions for the production of bioplastic. Under optimized fermentation conditions, Polyhydroxy butrate (PHB) was extracted and subjected to examine their properties via Thin Layer Chromatogram (TLC), Gas Chromatogram- Mass Spectrometer (GC-MS), Fourier Transform Infrared spectrum (FTIR). The presence of a brown spot in the TLC plate indicates the presence of hydroxyl group which is similar to the polymer group. GC-MS analysis of extracted PHB shows peaks at the retention time of 3.8, 11.6 which is corresponding to octadecanoic acid, hexadecanoic acid, butyl -2-ethyl ester confirms the presence of polymeric nature in the extracted PHB. The absorption bands of FTIR at 1719–1720 cm^{-1} indicate the presence of C=O group of PHB. The absorption peaks at wave numbers 500–1000 cm^{-1} , 1055 cm^{-1} and 1230 cm^{-1} denotes (OH) group, (C–O) stretch and (C=O) ester group. From these results, it was confirmed that the extracted PHB is having the potential to replace petroleum plastic.

Introduction

Synthetic polymers, “plastic” have become a necessary part of daily life with a wide range of applications in agriculture, household, medicine and packaging. Excessive use of petroleum derived plastic resulted in environmental pollution, since they remained in soil which poses threat to terrestrial and aquatic ecosystems. Most of the plastics available in the market are not easily degradable due to their petroleum nature. There is an urgent need to safeguard our environment from the plastic world in an eco-friendly way. In recent days, the use of biodegradable plastic seeks attention globally. Bio-plastics such as starch derivatives, poly lactic acid (PLA), polymeric cellulose, poly hydroxyl alkanoates (PHA) plays a wide role with the added advantage of being produced from renewable resources such as plants and microbial sources. There are many types of biodegradable plastics with different degrees of biodegradability. Among them, polyhydroxybutyrate (PHBs) are the only 100% biodegradable ones.

PHB is a biodegradable thermoplastic that can be extracted from a wide range of micro-organisms. PHB and its copolymers are members of the PHA family commonly known as best bacterial polyesters produced by a microbial process on enriched sugar medium and act as carbon and energy storage material. PHB synthesis takes place when a suitable carbon source is available in excess amount and cellular growth is limited due to other nutrient limitations. Bio-degradable polymers found to be a promising tool for reducing these global environmental issues.

Microbial PHB shares the common property with petroleum-derived plastics are represented in Table 1. This differentiates PHB from most other currently available biodegradable plastics, which are either water-soluble or moisture sensitive. PHB has good oxygen permeability, ultra-violet resistance but poor resistance to acids and bases. PHB is soluble in chloroform and other chlorinated hydrocarbons and biocompatible and hence it is suitable for medical applications also. Reshma *et al.* (2017). In reality, advancements in microbial synthesis, industrial-scale production and application are still far. A wide range of microbes was used in the production of PHB, which included bacteria, algae, actinomycetes,

some molds and yeast. Only a few were reported to accumulate a considerable amount of PHB, among 300 PHB-producing bacterial strains. (Wang, 2013). Both Gram-positive and negative bacteria accumulated PHB. But, Gram-positive *Bacillus* spp. are considered super microbial factories for commercial production of PHB was reported by Kumar *et al.*(2009).

Furthermore, the chemical structure of the PHB synthesized by the bacterial isolate was also determined by FTIR spectroscopy analysis.

Materials And Methods

The bacterial isolate *Bacillus siamensis* PHB 01 (MW440618) was selected based on the initial and secondary screening (Subasri *et al.*, 2021). Then the potent isolate was subjected to different fermentation conditions such as carbon source, pH, temperature, incubation period and characterized.

Cell cultivation and Extraction of PHB

For large-scale growth, inoculums were prepared in nutrient broth medium at 37°C and transferred to 500 mL of nutrient broth in a wide-necked 1 L culture flask, incubated at 37°C for 48h with continuous gentle shaking. A sample of culture solution (0.1-0.2 ml) was added to 4 ml of the sample were centrifuged for 30 min to precipitate the solution and then left for 24 h. 0.4ml of the sample was centrifuged for 30 min to precipitate the PHB. The solid pellet was resuspended and washed with 1ml portions of water acetone and ether. Chloroform was added and allowed to boil in a water bath at 100°C. The settled material after evaporation of chloroform was dried at 40°C for 30min. The extracted PHB was subjected to further characterization.

Characterization of PHB

The chemical structure and the thermal properties of PHB were used as parameters for qualitative analysis of PHB. Characterization and determination of native PHB like granules involved precise measurements to analyze their physical properties and were characterized by TLC, GC- MS, FTIR, XRD.

Thin layer chromatography (TLC)

A glass plate of 10 x 5 cm² size was coated with silica gel of about 3g per 15ml of chloroform. And the prepared solution was spread onto the plate using a spreader (Panda *et al.*, 2008). 1 µl of sample (Extracted PHB) was loaded on the TLC plate and allowed to run in the solvent system consisting of 1:1 of ethyl acetate and benzene mixture for 40 min. The plate was left to dry after the run and for staining 50 ml of iodine solution (Hi-media®) was vaporized in the water bath at 80 to 100°C. TLC plate was kept over the beaker containing iodine solution for 5-10 min to get it saturated with iodine vapor. The R_f values of the spots were calculated.

GC-MS Analysis

A solution of 1 ml chloroform, 0.85 ml of methanol and 0.15 ml of sulphuric acid was prepared. This solution was added to about 2mg of extracted PHB and heated to 100°C for 140 minutes. And the analysis was done at the Biocatalysts Laboratory, Department of Agricultural Microbiology.

Fourier Transform Infra Red spectroscopy analysis (FT-IR) of extracted PHB

FT-IR helps in identifying the chemical structure of the compound under study by finding the required functional groups. The extracted powdered PHB was subjected to IR analysis. The relative intensity of transmitted light was measured against the wavelength of absorption on the region 800 to 4000 cm⁻¹ using IR double beam spectrophotometer (Jasco, Japan). IR spectra of samples were measured at ambient conditions.

Results And Discussion

Climate change and environmental and waste management have turned attention to the development of biodegradable plastics with physical and chemical qualities equivalent to conventional plastics. There has been an increased demand in public as well as scientific research for the development of biodegradable polymers. PHB accumulation as cytoplasmic inclusions in certain bacteria during unbalanced growth conditions has proved to be the best suitable alternative to overcome this problem. More efforts to develop an economically feasible process for the synthesis of PHB should take the center stage most preferably from microbial origin. Based on the properties like biodegradable and nontoxic, it can be used for medical applications also. But the drawback in PHB lies in its cost of production, which is preferably high than petroleum plastic since PHB as it is currently produced cannot handle such high impact.

Many bacteria such as *Azotobacter*, *Bacillus*, *Archaeobacteria*, *Methylobacteria*, *Pseudomonas* have been found to synthesize PHA to varying levels. *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) has been the subject of much-published research work because it can accumulate PHAs up to 80 per cent dry weight Lee *et al.* (1996). In this regard, many *Bacillus* strains have been reported possessing the tremendous potential of PHB accumulation in their cytoplasm under nutrient limiting conditions at a level of 6–97% of dry cell weight. In this present work, attempts were made to characterize the PHB extracted from *Bacillus siamensis*

Detection by TLC:

The Chromatography Chamber was saturated with solvent system. The extracted PHB were dissolved in chloroform and spotted on TLC plate by capillary. The plate is carefully placed in a saturated chromatography jar and allowed to run. When the mobile phase reached 3/4th length, the plate was taken out and air-dried. Then, the TLC plate was exposed to iodine vapours. After a few minutes, brown coloured spot were observed which indicated the presence of lipids. Similar observations were made by Brigham *et al.* (2010). PHAs are primarily linear, head-tail polyesters composed of 3-hydroxy fatty acid

monomers Madison and Huisman (1999)]. Hence, from TLC, presence of lipids by the brown spot indicated the granules of PHB present in the bacterial strain.

GC-MS analysis:

Mostly the microorganisms accumulate either scl-PHA's with 3HB units or mcl- PHA's containing 3-Hydroxyoctate and 3hydroxydecanate as the major monomers. The major peak at 12.73 retention time represents the presence of 3-PHA. This retention time is on par with the results of Okwuobi *et al*, 2013. The peak at the retention time 3.8 depicted the presence of Octadecanodic acid which is the characteristic feature of PHB. Similar results were observed by Nurbas and Kutsal (2004). Kim *et al* . 2012 also referred to the same for the extracted PHB. The results of Bhuwal, *et al*. (2013), represented the presence of hexadenoic acid matches with the peak at the RT of 11.6 is matched with our retention peak at 11.8. Similarly the peaks at 12.792, 13.75 represent the tetradeconic acid and methyl ester respectively. And the monomers of the polyester family compounds were matched with the peaks at 13.75 and 15.72. The presence of ethyl, trimethyl groups shows the presence of many monomers, thereby providing crude polymer production.

FTIR

Data interpretation is in the form of stretching and bending of the peaks for the concerning compound i.e. PHB. The extracted PHB from *Bacillus siamensis* PHB01 was subjected to FT-IR analysis to find out the various functional groups that represent the signal peaks of PHB. Extracted PHB was subjected to IR analysis and absorption spectrum was recorded in the wavenumber range i.e. 4000 cm^{-1} to 400 cm^{-1} using a single beam spectrophotometer. The absorption spectra of the PHB from *Bacillus siamensis* PHB01 are shown in Fig. The absorption peak at wave number 3274 cm^{-1} represents hydroxyl group (-OH). Peaks at wavenumbers 2923 cm^{-1} , 2954 cm^{-1} correspond to methylene group. The peaks at wave numbers 1526 cm^{-1} , 1623 cm^{-1} and 2339 cm^{-1} correspond to (-C=C-) stretch, (N-O) asymmetric stretch and (-C≡C-) stretch respectively. The peaks at wave numbers 1376 cm^{-1} , 1450 cm^{-1} , indicate CH vibrations of methyl(-CH₃) and methylene (-CH₂) groups. The absorption peaks at wave numbers 500-1000 cm^{-1} , 1055 cm^{-1} and 1230 cm^{-1} denote (OH) group, (C-O) stretch and (C=O) ester group. The FT-IR analysis of PHB extracted from the isolate PHB01 correlated with the reports of Taran (2011) and standard.

The extracted polymer show peak at the wavelength of 2927.41 cm^{-1} , 1453.1 cm^{-1} , 1377.89 cm^{-1} 1242.9 cm^{-1} corresponding to specific rotations around carbon atoms specific to certain functional groups. The peak at 1242.9 cm^{-1} corresponds to -CH group. Similar results were obtained in *Pseudomonas putida* by Asheeba *et al*. (2013). The peak at 1242.9 indicates the C-O-C group and it is on par with the results of Nygaard *et al*. (2019). The presence of functional groups like CH₂, CH, C=O and -OH present in the sample PHB01 revealed the character of extracted PHB. Pradhan Shreema, 2014 also obtained similar results. The methionine group (CH) gave a strong bond in the range of 1300- 1400 cm^{-1} and 2900 to 3000 cm^{-1} respectively. These frequencies values 29.27.41, 1453.1, 1377.89 cm^{-1} were higher than the

normal values because of the polymerization. The carbonyl group (C=O) gave a strong bond in the range of 1636-1673 cm^{-1} where the extracted PHB showed the peak at 1640.16 cm^{-1} Varda *et al.* (2014).

The C-O group showed strong and broad absorption in the range of 1047- 1089 cm^{-1} . Here the strong and broad absorption was observed at 1076.08 cm^{-1} . The C-H stretch bonds in the polyester were assigned to the bands located in the spectral region around 2900 cm^{-1} . The obtained FTIR absorption peaks from the culture *Bacillus siamensis* PHB01 are in agreement with the corresponding spectra to commercial PHB. Based on the above results, it was concluded that the extracted compound from the isolate PHB01 showed be PHB. Similar results were obtained in the bacteria *Cuprivirudus necator* ATCC 17697 Nygaard *et al.* (2019). The polymer accumulated in the cytoplasm of the PHB01 in the form of granules showed an FTIR spectrum corresponding to the previous studies.

Conclusion And Future Approach

Bacillus siamensis PHB 01 is capable of producing PHB. Thus by utilizing the optimum culture conditions we can solve the problems of the high cost of PHB production. To deal with ever-increasing plastic pollution, PHB from bacterial sources using renewable carbon is the better alternative. In this research, PHB produced by *Bacillus* sp. PHB01 is crystalline in nature and biodegradable. PHB produced by the bacterial system is brittle and breaks easily. Thus, blending PHB with other polymers in an economic way improves its mechanical properties. In both ways the use of starchy material as a carbon source for PHB production and polymer for blending.

Declarations

Authors contribution:

All the authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Subasri, Mani , Gomathi Velu and Kavitha Mary Jackson . The first draft of the manuscript was written by Subasri Mani, Gomathi Velu and Kavitha Mary Jackson were rewritten the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of interest:

The authors have no conflict of interest.

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Tables

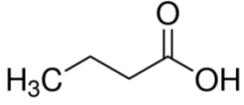
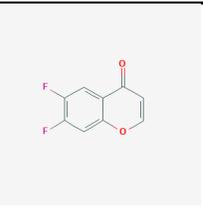
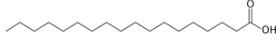
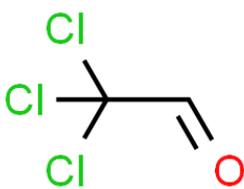
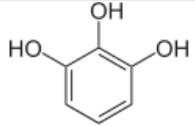
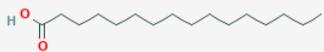
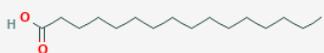
Table 1: Common Properties of PHB (modified from Park *et al.*, 2001)

Water Insoluble	Soluble in Chlorinated Hydrocarbons Chloroform
Good Oxygen Permeability	Relatively Resistant to Hydrolytic Degradation
Susceptible To Acids And Bases	Resistant to Ultra Violet
Non-Toxic	Biocompatible
High Tensile Strength (40mpa)	Melting Point of 179 °C
Extremely Crystalline (80%)	Thermoplastic Process Ability
Curvature coefficient (Young) (GPa) - (4-3.5)	Density g/cm ³ (1.25-1.23)
Glass transition Temperature (15-10)	Molecular weight M.wt. (Dalton)- ⁵ 10 ×8-1

Table 2 : Nutrient Agar Media

Components	g/L
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Agar	20
Distilled Water	1000 mL
pH	7.4 ± 0.2

Table 3: GC- MS Analysis Report

Retention Time	Compounds Identified	Chemical Structure	Chemical Formula	Desirable Use
3.2	Butanoic Acid		$C_4H_8O_2$	Used for plastic making
3.8	2-ethyl 2,3,3-Trimethylbutanoic acid		$C_9H_{18}O_2$	Used for plastic making
4.439	H-1-Benzopyran-4-one		$C_{21}H_{20}O_{10}$	Used for plastic making
4.6	Dososanedioic acid, Dimethyl ester, Octadecanoic acid		$CH_3(CH_2)_{16}COOH$	Used for plastic making
7.235	C-Chlorol-2-butryothienone		C_2HCl_3O	Used for plastic making
10.291	Benzenetriol		$C_6H_3(OH)_3$	Used for plastic making
11.807	Hexadecanoic Acid		$C_{16}H_{32}O_2$	Used for plastic making
12.792	Hexadecanoic acid		$C_{16}H_{32}O_2$	Used for plastic making
13.75	Octyl-, methyl ester		$C_{19}H_{36}O_3$	Used for plastic making

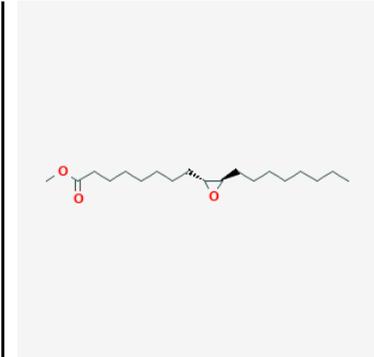
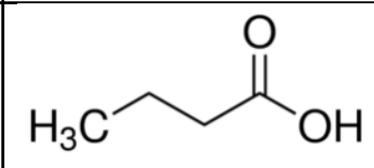
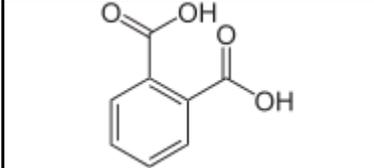
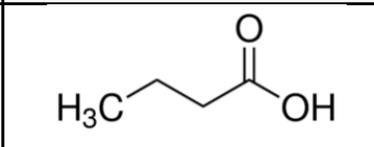
				
15.72	Octadecenoic acid		$C_{18}H_{34}O_2$	Used for plastic making
20.575	Benzedicarboxylic acid, Butyl-2-ethylester		$C_{11}H_{10}(CO_2H)_2$	Used for plastic making
23.316	Methylester methylether 3-hydroxybutyric acid		$CH_3CH_2CH_2CO_2H$	Used for plastic making

Table 4: IR spectrum of sample

Characteristic peaks cm^{-1} of Extracted PHB	Reference Peaks	Intensity	Description
2927.41	~2900	Strong	C-H Vibration
1640.16	1635	Strong	Carbonyl group (C=O)
1453.1	1457	Strong	CH ₃ Asymmetric deformation
1377.89	1379	Strong	CH Symmetric deformation
1242.9	1276	Strong	CH group
1076.08	1076	Strong	Ester group (C-O)
1022.09	1029	Strong	C-O polymeric group
495	491	Strong	Alkyl Halides

Figures



Figure 1

Extraction of PHB



Figure 2

Extracted PHB



Figure 3

Extracted PHB used for analysis

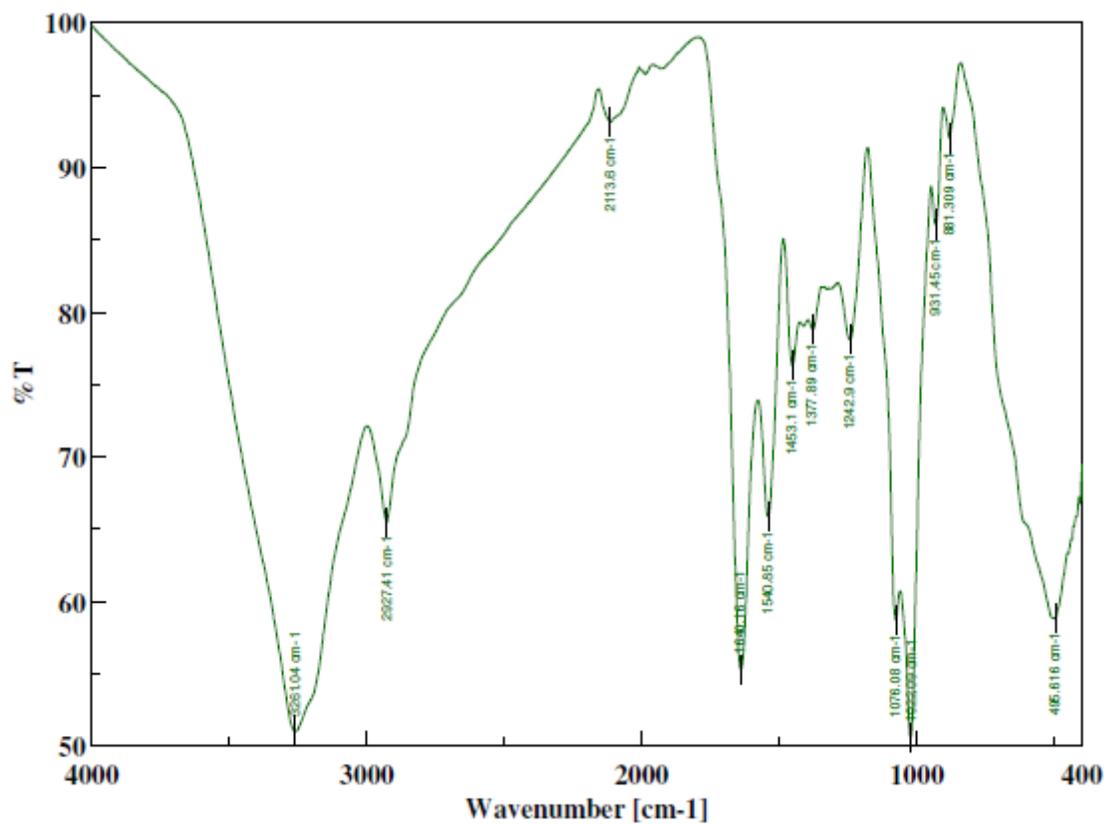


Figure 4

FTIR spectrum

[Comments]
 Sample name
 Comment
 User
 Division
 Company

SAMPLE-I 1003

TNAU

[Measurement Information]
 Model Name FT/IR-6800typeA
 Serial Number A001661794

[Detailed Information]
 Creation date 3/10/2021 4:43 PM

Measurement Date 3/10/2021 4:41 PM

Data array type Linear data array
 Horizontal axis Wavenumber [cm-1]
 Vertical axis %T
 Start 349.053 cm-1
 End 4000.6 cm-1
 Data interval 0.964233 cm-1
 Data points 3788

Light Source Standard
 Detector TGS
 Accumulation 15
 Resolution 4 cm-1
 Zero Filling On
 Apodization Cosine
 Gain Auto (8)
 Aperture Auto (7.1 mm)
 Scanning Speed Auto (2 mm/sec)
 Filter Auto (10000 Hz)

TAMILNADU AGRICULTURAL UNIVERSITY - AGRICULTURAL MICROBIOLOGY

INSTRUMENT : PERKIN ELMER CLARUS SQ8C
INJECTION : 1 MICRO LITER
SAMPLE ID : SAMPLE-15 0503

COLOUMN : DB-5 MS CAPILARY STANDARD NON POLARCOLOUMN
DIMENSION : 30Mts, ID: 0.25 mm, FILM: 0.25 IM CARRIER GAS: He

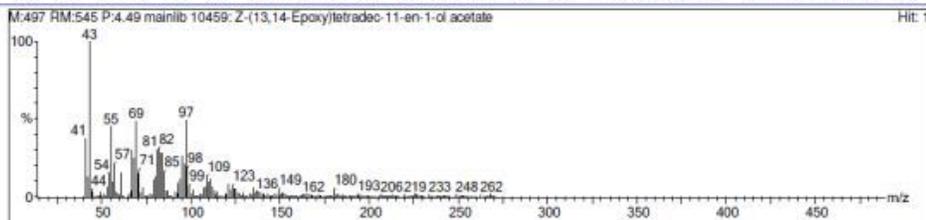
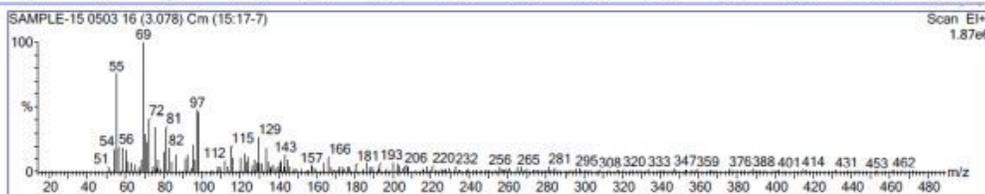
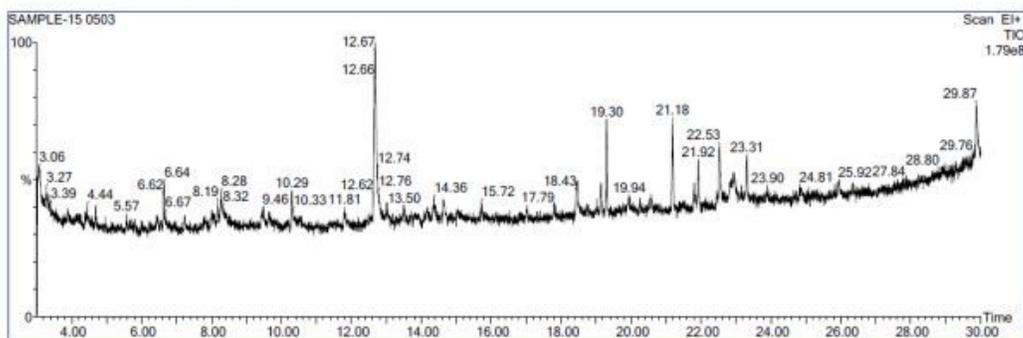


Figure 5

GC-MS spectrum

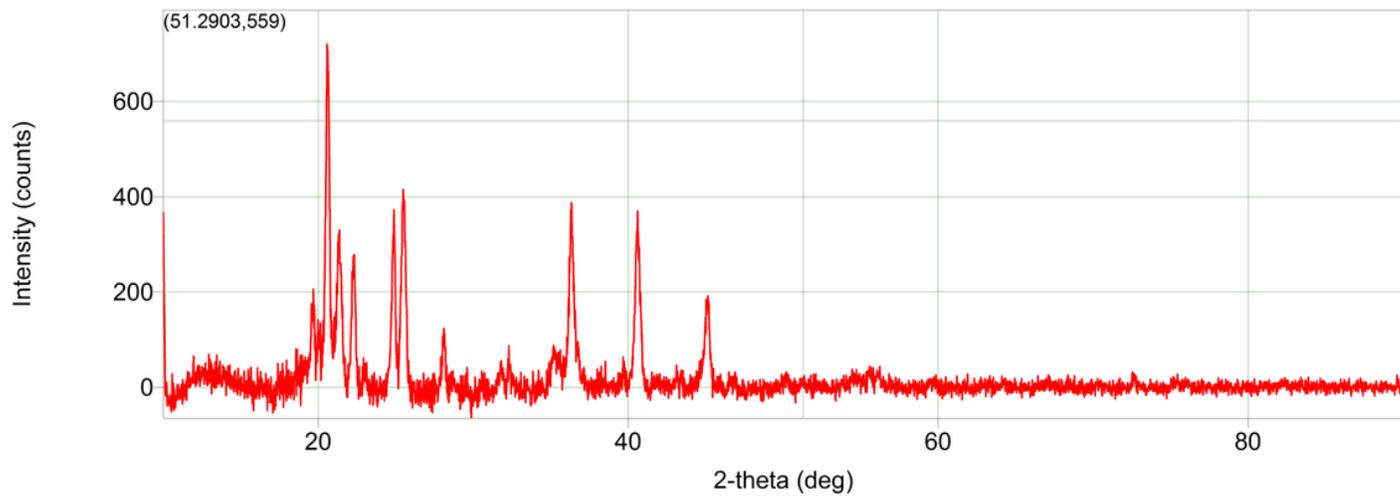


Figure 6

XRD Graph