ISSN : 0974 - 7435 Volume 7 Issue 2

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 7(2), 2013 [41-48]

Bioutilization of organic waste in the production of poly hydroxy alkonate

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ABSTRACT

Polyhydroxyalkonates a family of polyesters is accumulated as granules in the cells of *Pseudomonas putida* was significantly depended on the ratio of carbon and nitrogen in the culture medium. The presence of PHA in Pseudomonas putida was confirmed by sudan Black and Nile Blue staining. Kitchen waste and oil waste were used as the renewable raw material for the production of PHA. Highest production of 7.27% was seen in oil waste and 6.3% in vegetable waste. The purified PHA was further confirmed by IR and NMR which showed the presence of the functional groups CO and OH which correlated with the control PHA.

KEYWORDS

PHA;
Renewable resources;
Sudan black;
Bioplastics;
Pseudomonas putida.

INTRODUCTION

In response to problems associated with plastic waste and its effect on the environment, there has been considerable interest in the development and production of biodegradable plastics (Bioplastics)[25]. Biotechnological processes are being developed as an alternative to existing route or to get new biodegradable biopolymers. Bioplastics are known as biodegradable plastics. Biodegradation is a process that describes the mineralization of organic structures by microorganisms. These microorganisms convert the bioplastics in to carbon-di-oxide, methane, water and biomass.

In order to make the production of PHAs economically more attractive, the use of inexpensive substrates has been investigated thoroughly[5].

The most common raw materials used in the production of biomass-based plastics are corn, starch and potatoes, including the biomass fraction present in waste (from households, municipal waste, dairy industry, paper mills, forestry etc.). About 150 different hydroxyalkanoic acids have been identified as constituents of bacterial polyesters[23]. The first Polyhydroxyalkanoate, a homopolymer poly-β-hydroxybutyrate was discovered by Lemoigne in 1925[12]. Polyhydroxy alkanoates are a class of natural polyesters which can be produced and accumulated as intracellular and energy storage compounds by many Gram-positive and Gram-negative bacteria from at least 75 different genera depending on the types of carbon sources available and the biochemical pathways that are operating in the cell.[13] This polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of en-

ergy storage material accumulated intracellularly to be metabolized when other common energy sources are not available[6,15]. Some bacteria may accumulate huge quantities (up to 80% of dry weight) of polyhydroxyalkanoate[14]. The microbial polyester is a crystalline thermoplastic with properties comparable to that of polypropylene[2].

The granules of Polyhydroxy alkanoates stored within the cells are clearly visible under electron microscope. Under Transmission Electron Microscope (TEM), it appears as electron-dense bodies. In terms of molecular weight, PHA can weigh between 2 X 10^5 to 3 X 10^6 Daltons[8]. The precise value depends on the microorganisms and its growth conditions[4]. Native Polyhydroxyalkanoates granules can be stained with Sudan Black B[3], Nile Blue A[17] and Nile Red. PHAs are more specifically stained by Nile Blue A, where its presence is indicated by strong orange fluorescence.

The rate of polyhydroxyalkanoate accumulation can be increased by increasing the ratio of carbon source to nitrogen source[16]. Among all, polyhydroxybutyrate (PHB) that consists of only 3-hydroxybutyrate monomer is the most commonly found polyhydroxyalkanoates in the nature[11]. Polyhydroxybutyrate has physical properties that resembled synthetic petrochemical-based plastics. It can be used as a biodegradable thermoplastic material for waste management strategies and biocompatibility in the medical devices.

Polyhydroxy alkanoate synthase is the key enzyme of Polyhydroxy alkanoate biosynthesis and catalyze the conversion of 3-hydroxyl-CoA substrates into Polyhydroxyalkanoates with the concomitant release of CoA[18].

Approximately 300 different bacteria, encompassing Gram-negative and Gram-positive species have been reported to accumulate various Polyhydroxyalkanoates[22].

A number of bacteria including *Alcaligens eutrophus, Alcaligens latus, Azotobacter vinelandii, Methylotrophs, Pseudomonas* and recombinant *Escherichia coli* have been employed for the production of Polyhydroxyalkanoates and the productivity greater than 2 g PHA /L/h has been achieved[19].

Polyhydroxyalkanoate have been widely used in the development of cardiovascular products such as pericardial patches[7] patch material in the pulmonary circulation[24] and as vascular grafts[10]. Impressive results have been obtained in the development of cell-seeded tissue engineered heart valves using Polyhydroxyalkanoate such as (3-hydroxyoctanoate) and Polyhydroxybutyrate[9].

Polyhydroxyalkanoates are also a potential material for applications in controlled drug release systems[21]. The biocompatibility and biodegradability properties of Polyhydroxyalkanoate make them attractive as materials for drug delivery.

In the present study the rate of production of polyhydroxy alkonate by microorganisms using the waste product as the source was evaluated.

MATERIALS AND METHODS

The microorganism capable of producing Polyhydroxyalkanoate was isolated and identified from the soil sample.

The garden soil was collected from the Dumpyard soil, Pallikaranai. For the isolation, the serial dilution technique was followed.

Isolation and characterization of microorganism

The organism grown in the nutrient agar was picked out and streaked individually. Individually isolated strain was screened for the presence of Polyhydroxyalkanoate by Sudan Black staining[20] and Nile Blue staining[17]. The positive strain in Nile Blue and Sudan Black staining was subjected to Biochemical identification studies.

Production of polyhydroxyalkanoates

Three different types of sources were used for Polyhydroxyalkanoate production.

(a) Production of polyhydroxyalkanoate in PHA medium, oil waste and kitchen waste

Pseudomonas putida was inoculated in Polyhydroxyalkanoate medium, oil waste medium (50g of coconut oil cake and 50g of sesame oil cake, boiled, filtered), Kitchen waste medium (100g of kitchen waste chopped, boiled, filtered). After the period of 3 days at 37°C incubation, Polyhydroxyalkanoate was isolated and used for analysis.

Extraction of polyhydroxyalkanoate

After the period of incubation bacterial cells were

harvested by centrifugation at 10000 rpm for 10minutes at 10°C. Pellets were washed twice with distilled water. Cells were dried at 105°C for 24 hours in hot air oven and then cooled. To the dried cells 20ml of 30% sodium hypochlorite and 20 ml of chloroform was added. The suspension was incubated at 30°C for 90 minutes in shaking incubator. The mixture was then centrifuged for 20 minutes. The lower phase was washed twice with distilled water and Polyhydroxyalkanoates were precipitated with ethanol. The precipitate was dried and weighed. The extracted Polyhydroxyalkanoate was dried and its weight was recorded.

Determination of polyhydroxyalkanoates contents in cells

The content of polyester in the cells was calculated as the ratio of weight of extracted Polyhydroxyalkanoate to the cell dry weight, from which the Polyhydroxyalkanoates were extracted[25].

Analysis of polyhydroxyalkanoates

(a) Fourier transform-Infrared spectroscopy (FT-IR)

FTIR was recorded to determine the functional group present in the polymer. FTIR spectrum was taken using PERKIN ELMER spectrum one in the range of 400-4000 cm-1.

(b) ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR)

NMR was recorded to calculate the number of carbon and hydrogen atoms present in the polymer. ^{1}H and ^{13}C NMR spectra were obtained with BRUKER AVANCE III 500 MHZ NMR system.

RESULTS AND DISCUSSION

The use of Polyhydroxybutyrate as a substitute for non-biodegradable petroleum based plastics cost substantially more than its fossil fuel based counterparts and offer a number of performance advantage other than biodegradability.

Isolation of polyhydroxyalkanoate producing microorganisms

About five microorganisms were isolated from soil samples out of which only one strain was sudanophilic as confirmed by Sudan Black staining. (Figure 1) and showed characteristic orange fluorescence, with Nile Blue A stain indicating the possible presence of Polyhydroxyalkanoates in the cells. (Figure 2)

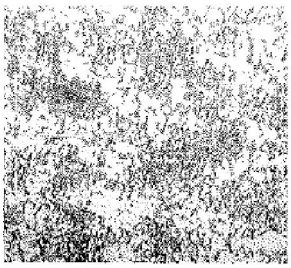

Figure 1 : Sudan black staining for strain1

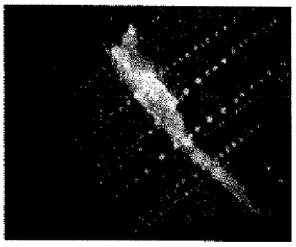

Figure 2 : Nile blue staining for strain1

Screening of organism

Based on the biochemical characteristics the isolate was characterized, identified and confirmed by Bergy's manual as Gram-negative *Pseudomonas putida.*

Production of polyhydroxyalkanoates from oil cake, kitchen waste, PHA medium

Oil cakes are by products obtained after oil extraction from the seeds. Oil cakes are utilized as a potential raw material in bioprocess as they provide an excellent substratum for the growth of microorganism supplying the essential nutrients to them. One of the limiting factors for the commercialization of Polyhydroxyalkanoates is the high cost of the carbon substrates used for the production. The waste materials generated from kitchen, when thrown away, creates major environmental problems. Hence the production of the polymer from kitchen waste, the cost effective substrates rich in different carbon components appeared feasible for Polyhydroxyalkanoate production. As control, the production of Polyhydroxyalkanoate was studied in PHA medium. The medium has acted as an artificial source containing carbon source in the form of glucose.

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TABLE 1 : Polyhydroxyalkanoate production by *Pseudomonas putida*e in oil cake extract, kitchen waste, PHA medium

Source	Cell Dry Weight (g/L)	PHA Concentration(g/L)	PHA Content (%)
Oil cake	1.086	0.158	7.27
Kitchenwaste	1.047	0.132	6.30
PHA medium	1.052	0.040	3.84

The data reveals that % of PHA (in terms of cell mass) is higher in oil cake extract medium than the kitchen waste and PHA medium. The coconut and sesame oil cakes have high protein content (supplement of nitrogen). In present study good yield has been seen even in the increased presence of nitrogen. The reason might be predicted as the nitrogen source supplied through the oil cake has increased the protein synthesis of the organism and increased the biomass to 1.086 g/L for *Pseudomonas putida*. The organism has utilized the residual fatty acids present in the oil cake to produce Polyhydroxyalkanoates (through β-oxidation).

Comparing the other source medium (oil cake and kitchen extract medium); the yield of the Polyhydroxyalkanoate in PHA medium is low, even though the production is seen. Hence, it can be predicted that the appropriate nutrients supplied in the medium has not initiated for the increased production of Polyhydroxyalkanoate.

After extraction of Polyhydroxyalkanoate by chloroform, the Polyhydroxyalkanoate appeared as sheet. (Figure 3,4,5) shows the photographs of the thin film sheets of Polyhydroxyalkanoate.

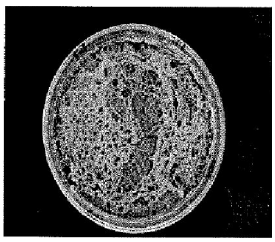

Figure 3 : PHA exracted by *Pseudomonas putida* from oil cake extract

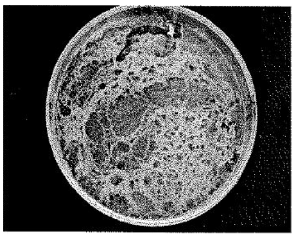

Figure 4 : PHA exracted by *Pseudomonas putida* from kitchen waste

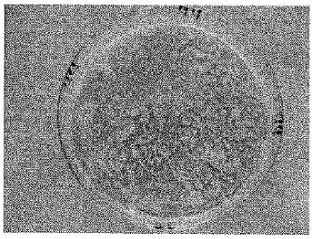

Figure 5 : PHA exracted by *Pseudomonas putida* from PHA medium

IR

The IR spectrum (TABLE 2, 3, 4, 5) of polymers extracted in this study was recorded in the range of 400-4000 cm-1 and It showed the marked peaks at numbers ranging (3434-3465), (2922-2926), (1451-1465) and (1055-1069) representing the presence of OH bonding, presence of aliphatic CH stretching, C=O and C -O groups which resembles the functional group present in the commercial poly hydroxyl butyrate and hence the isolated compound is predicted to be Poly hydroxyl butyrate.

TABLE 2 : IR peak regions for PHA extracted from oil cake medium at pH 7.0

Microorganism	Peak region	Comments
Pseudomonas putida	3435	Intramolecular H bonding
	2924	CH_2 stretching
	1745	C = O
	1057	C-O stretching

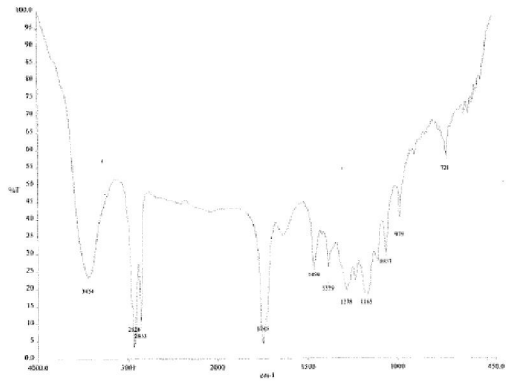

Figure 6 : IR peak for polyhydroxyalkanoates produced by *Pseudomonas putida* in oil cake extract medium

TABLE 3 : IR peak regions for PHA extracted from kitchen waste medium at pH 7.0

Microorganism	Peak region	Comments
Pseudomonas putida	3434	Intramolecular H bonding
	2924	CH_2 stretching
	1745	C = O
	1057	C-O stretching

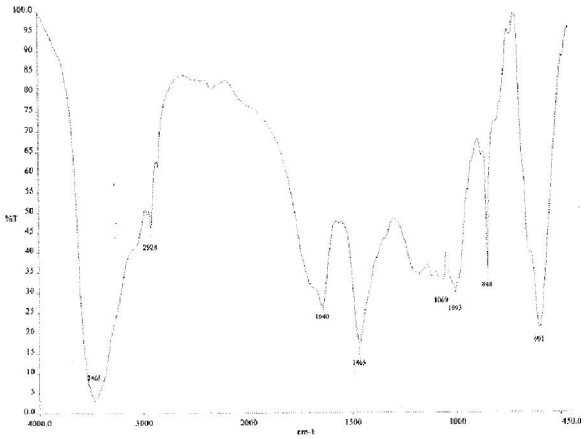

Figure 7 : IR peak for polyhydroxyalkanoates produced by *Pseudomonas putida* in kitchen waste extract medium

TABLE 4 : IR peak regions for PHA extracted from PHA medium at pH 7.0

Microorganism	Peak region	Comments
Pseudomonas putida	3465	Intramolecular H bonding
	2924	CH_2 stretching
	1648	C = O
	1069	C-O stretching

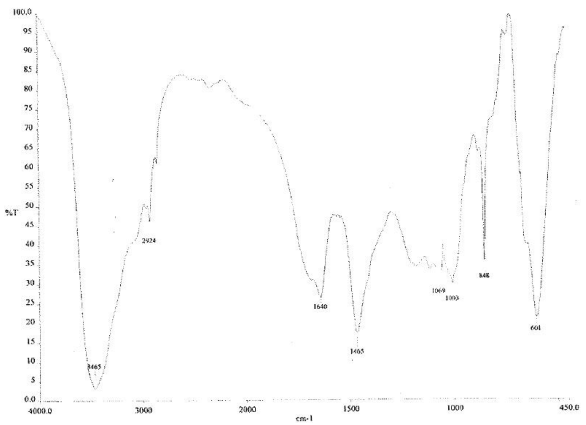

Figure 8 : IR peak for polyhydroxyalkanoates produced by *Pseudomonas putida* in PHA medium

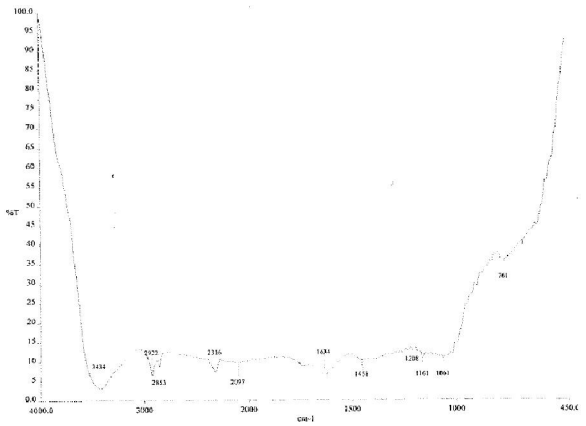

Figure 9 : 1R peak for commercial polyhydroxyalkanoates

TABLE 5 : IR peak regions commercial PHA

Control	Peak region	Comments
	3435	Intramolecular H bonding
	2922	CH_2 stretching
	1634	C = O
	1061	C-O stretching

NMR

^{1}H NMR spectrum indicates that signal characteristic of PHA was observed in the polymer extracted from different carbon sources containing media as a doublet at 1.29 ppm which is attributed to the methyl group, a doublet of quadruple at 2.5 ppm which is attributed to methylene group and a multiplet at 5.28 ppm characteristic of methylene group.

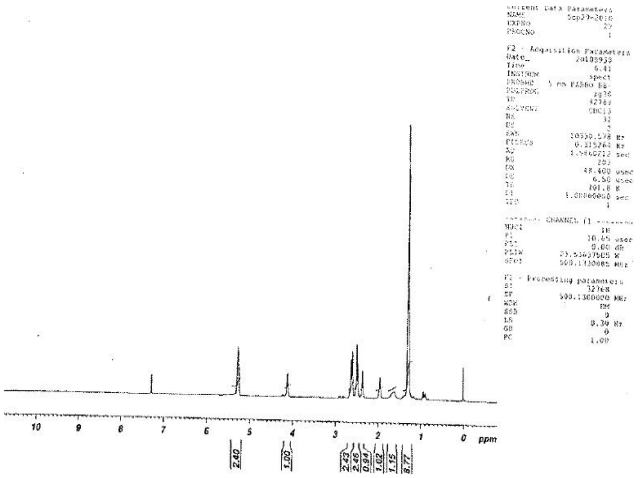

Figure 10 : ^{1}H NMR spectrum for polyhydroxyalkanoates produced by *Pseudomonas putida* in oil cake extract medium

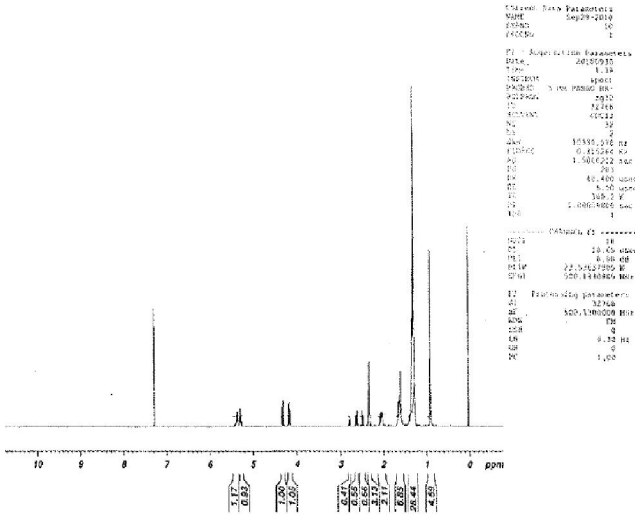

Figure 11 : ^{1}H NMR spectrum for polyhydroxyalkanoates produced by *Pseudomonas putida* in kitchen waste extract medium

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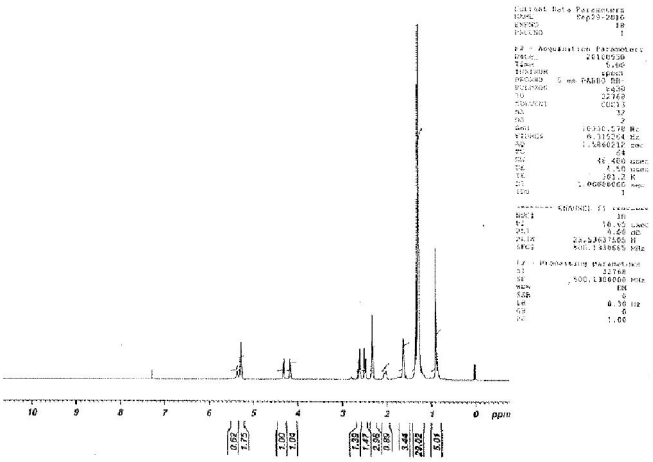

Figure 12 : ^{1}H NMR spectrum for polyhydroxyalkanoates produced by *Pseudomonas putida* in PHA medium

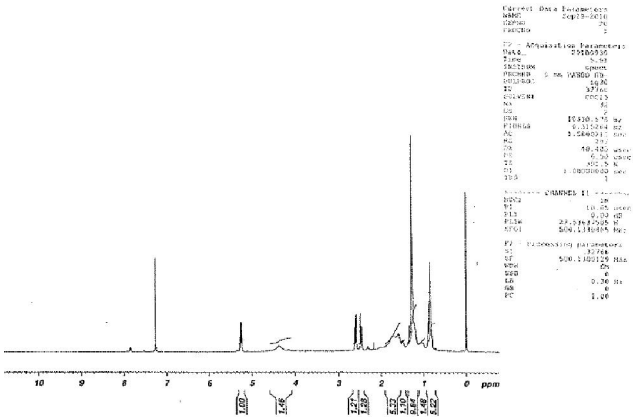

Figure 13 : ^{1}H NMR peak for commercial PHA

^{13}C NMR spectrum of the compound was recorded in the range of (0-200) ppm. The spectrum of the biopolymers showed transitions at near enough positions to ensure that chemically all the polymers were similar. Assignment could be done for the presence of CH_3 (~23 ppm), CH_2 (~45 ppm), CH (~77 ppm) and CO (~168 ppm) groups.

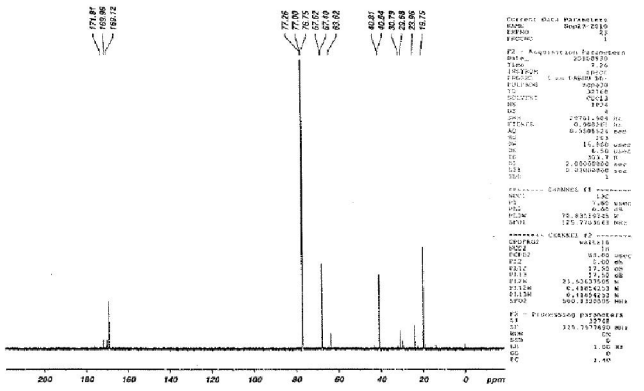

Figure 14 : ^{13}C NMR spectrum for polyhydroxyalkanoates produced by *Pseudomonas putida* in oil cake extract medium

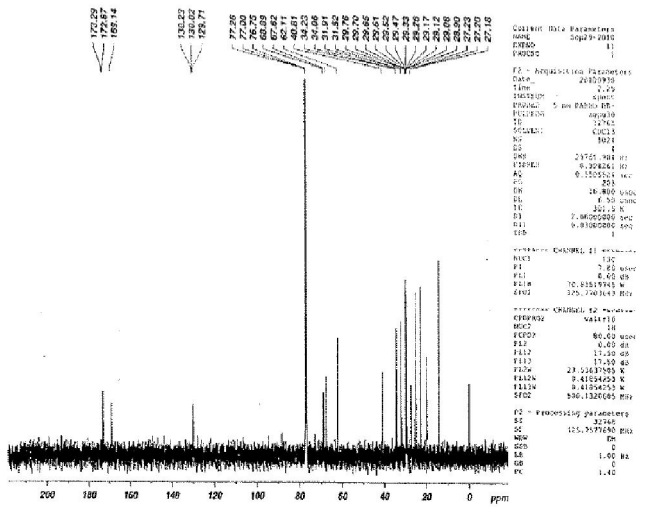

Figure 15 : ^{13}C NMR spectrum for polyhydroxyalkanoates produced by *Pseudomonas putida* in kitchen waste extract medium

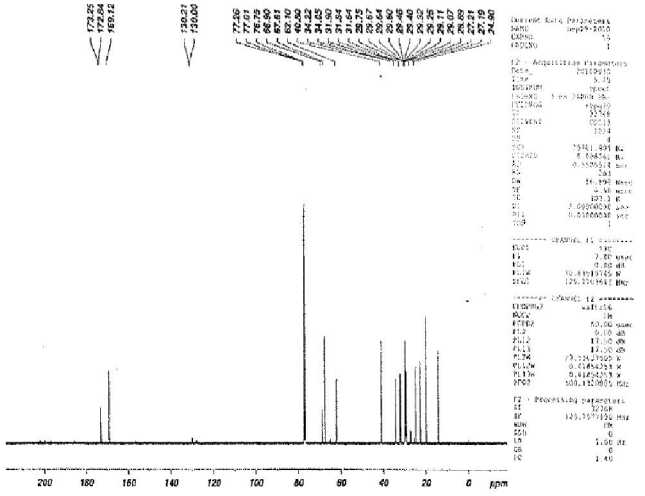

Figure 16 : ^{13}C NMR spectrum for polyhydroxyalkanoates produced by *Pseudomonas putida* in PHA medium

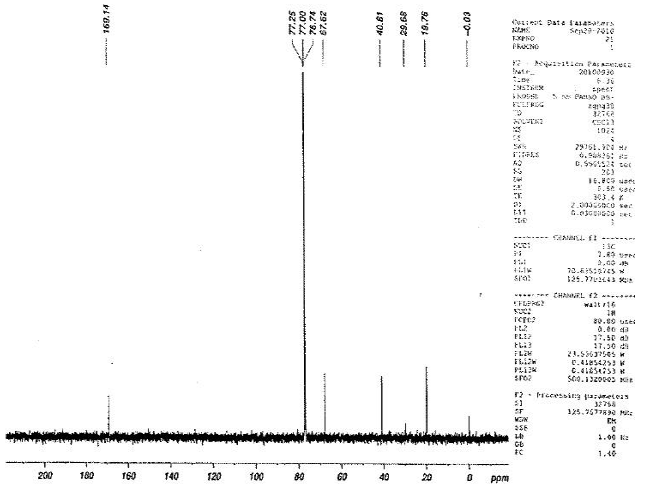

Figure 17 : ^{13}C NMR spectrum for commercial PHA

The spectrum analysis was compared with other previous references and found to be similar with PHB[1].

CONCLUSION

Research in the production of Polyhydroxyalkanoates as a petrochemical alternatives for the future has been explored using bacteria and waste products. Hence, the waste materials which was considered as one of the main factor of pollution was diverted to produce Polyhydroxyalkanoate, an alternative source of petroleum based plastics. Thus the word pollution to a certain extent can be nullified by this present study.

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