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Production of polyhydroxyalkanoates (PHA) by *Bacillus subtilis* ANM1 using bio effluents as substrates and characterization using FTIR analysis

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ABSTRACT

Polyhydroxyalkanoates are Biopolymers produced by many bacterial species present in nature. These are degradable compared to conventional plastics and are produced as intracellular reserve granules at the time of starvation like depletion of nitrogen source, salt stress and excess of carbon source. However, commercialization of such bio degradable polymers has not been seriously attempted due to several reasons and one such reason is that PHA is not flexible as expected. Moreover PHA cannot be produced easily at low cost as the carbon source used for the media preparation is very expensive. This led to the usage of agro industrial wastes as a replacement for sucrose as an energy source in the media. In this study, different agro industrial residues were used as cheaper source of carbon like sugarcane molasses, distillery effluent, milk whey from diary industry, paper industry effluent, and biodiesel industry generated crude glycerol waste. PHA was extracted using Sodium hypochlorite extraction from *Bacillus subtilis* ANM1 and the extracted PHA from different industrial sources was characterized using Fourier Transformation Infra Red (FTIR) spectroscopic technique and checked for the functional groups. The polymer produced from all the agro industrial sources showed characteristic bands for different functional groups of PHA like CH, CH_2, C=O, and C-O. Their frequency value was found to be much higher than that of the normal. The carbonyl group showed strong band in 1636-1673 cm^{-1}. The C-O group showed a strong absorption in 1047-1089cm^{-1}. Thus the study suggested that bio-effluents could be used for the production of Biopolymers so that it acts as a cost effective method for polymer extraction.

KEYWORDS

Polyhydroxyalkanoates;
Fourier transform infra red;
Sodium hypochlorite;
Molasses;
Bacillus subtilis ANM1.

INTRODUCTION

Plastics are kind spectacular inventions of science and technology as they are easily moldable and can be changed into varying forms and shapes. They have engrossed human life in almost all fields like Agricultural industry for delivering the pesticides or fertilizers, Medicinal industry for making artificial bones and sutures, Cosmetic and domestic industries for making various products like films, sheets, molds and casts. But the long term ill use of plastics has led to the accumulation of plastics in the environment. They make the soil and water polluted and prevents seepage of useful materials which is necessary for the balance of the ecosystem.

The PHA is produced as polymers within the bacterial cells and it is stored as intracellular reserve granules at the time of starvation. They have numerous flexible mechanical properties which make them find diverse applications. Apart from these properties, the biodegradability and biocompatibility of PHA shows that they are non toxic and can be used for various biomedical applications. It is possible to tailor the structures of PHA by changing the composition of the media used and by genetically altering the pathway of PHA production[5].

PHAs are of different types based on the number of carbon atom in them varies. These variations results in changes in the carbon skeleton, which offers PHA numerous versatile properties[9].

In the present investigation, the wastewaters from agro based industries were directly used for production of PHA as they are found to contain enough nutrients needed for microbial growth. This method has proposed as a significant cost effective technique in PHA extraction process as the cost of the carbon source used is addressed as the major problem in large scale manufacture of PHA.

MATERIALS AND METHODS

Microorganism

The *Bacillus subtilis* ANM1 was obtained from soil samples collected in and around Coimbatore. The mother culture was grown at 30°C and pH value 7.0 in Nutrient Broth [Yeast extract 2.0(g/L); Tryptone 5.0(g/L); Sodium chloride 5.0(g/L)] and maintained at 4°C on Nutrient agar plates. The culture was sub cultured every 2 weeks to maintain purity.

Parasporal body analysis by sudan black staining

The presence of intracellular granules was confirmed by staining the cells with Sudan black dye. The 48hours old *Bacillus* culture was used to make thin smear on a glass slide and then fixed on slide using heat fixation technique. The heat fixed slide was immersed in a filtered solution of 0.3% (w/v) Sudan black (in ethanol) for 20 min. Then the slide was then completely dipped inside a beaker containing Xylene solution and blot dried with absorbent paper. Finally, the microscopic slide was counter-stained for 10 s with (0.5% w/v) aqueous safranin. The slide was then rinsed with tap water and blot dried and examined under a microscope.

Shake flask cultivation condition using agro industrial residues as carbon source

The 24 hour old *Bacillus subtilis* ANM1 culture from NB was centrifuged at 10,000rpm for 20 minutes and transferred into a medium(pH 7.0) containing different agro industrial wastes and by products like sugarcane molasses, distillery effluent, milk whey, paper industry waste, crude glycerol and coke industry waste.

PHA recovery by chloroform extraction

The cells obtained from all the sources were first treated with diluted sodium hypochlorite solution. The solution was then centrifuged at 6000X g for 10 min, to get the pellets, which was treated with acetone and chloroform. From the two phases obtained, the lower chloroform containing phase was taken and separated using precipitation technique.

Sample preparation for FTIR analysis

The Polymer so obtained was evaluated for functional groups by using FTIR spectrum. Approximately 2 mg of the extracted polymer was mixed thoroughly with KBr pellets and the spectrum was recorded in a FTIR spectrometer from 400 to 4000cm^{-1} range.

RESULTS

The Sudan Black stained slide was examined under a microscope. The prominent dark PHA granules were clearly visible as the cells were enlarged in size indicating the presence of inclusion bodies within them.

The PHA extracted was evaporated and dried for the FTIR study. It was confirmed that the strain was able to utilize biowastes as carbon sources suggesting that agro industrial wastes can be used as a feed material for biopolymer production. The amount of PHA produced using molasses, distillery spent wash, milk whey, coke industry waste, and glycerol were 2.50g/l, 0.46g/l, 0.88g/l, 0.75g/l, and 0.85g/l respectively. However the quantity of polymer produced varied with the carbon sources used.

Of the different substrates used, the paper industrial waste did not produce biopolymers as the strain was not able to utilize cellulose as carbon source[7].

The IR spectrum of the compound extracted is shown in the TABLE with the frequency expressed in cm^{-1}, the bond names and the functional group representing the wavenumber.

The numerals in the brackets indicate the peaks representing the functional groups in the corresponding graphs shown below.

The IR spectrum analysis gave insights to the chemical structure of the polymer without any breakage of bonds. PHAs with different backbones and different chemical groups are previously studied[10].

FTIR spectrum of PHA synthesized from NB medium

The spectrum of FTIR revealed the presence of marked peaks at wave numbers 3440.77cm^{-1} to 3703.07cm^{-1}, 1600 cm^{-1} and 1724.10 cm^{-1} corresponding to the hydroxyl (-OH) stretching[6], C=O and aliphatic stretching of carbonyl group of RCOA of that of the polymer[4]. Other absorption bands at 1379.93 cm^{-1} and 1454.36 cm^{-1} were that of the aliphatic $-CH_3$, $-CH_2$ groups. The band at 1228.03 cm^{-1} to 1279.56 cm^{-1} were characteristic to that of Carbon oxygen stretch of the esters(C-O-C bond)[6]. The strong band at 922 cm^{-1} was predicted to be that of alkyl stretch[3].

TABLE 1 : FTIR spectrum (wavenumber in cm^{-1}) chart of PHA from different nutrient sources

Medium	Molasses	Distillery Effluent	Whey	Coke industry waste	Crude Glycerol	Bond names	Functional groups
			1238.357	1000(1) 1125(2) 1150.20(3)	1281.64(1)	Carbonyl stretch vibration	-C-O
1228.03(2) 1279.56(3)	1279.931(1)		1245.78(1)1319.21(2)			Carbon oxygen carbon stretching of esters	C-O-C
922.56(1)	1381.55(2)	1281.646(1)	2925.59(6)		1276.93(2)	Carbon hydrogen stretching	-CH
1379.93(4)			1375.22(3) 1419.52(4)	1379.93		Methyl vibration	$-CH_3$
1600(6)	1650.953(3)	1688.52(2)		1460.08		Carboxylic stretch vibration	C=OO
1724.10(7)	1740.53(4) 1850.536(5)	1724.38(3)	1735.81(5)	1727.1(4)	1726.29(3)	Carboxyl stretch of ester	C=O
1454.36(5)				2948.02(5)	2931.59(4)	Aliphatic stretching	$-CH_2$
3440.77(8)			3497.67(7)	3422.02	3749.36(5)	Hydroxyl stretching	-OH

PHA from medium amended with molasses as carbon source

The bands found at 1228.643 cm^{-1} to 1279.93 cm^{-1} corresponds to the carbon oxygen carbon stretch, but the band at 1740.53 cm^{-1} shows the C-O bonds of esters[4]. The stretch at the region 1850 cm^{-1}, were the bands due to the carbonyl vibrations of the polymer[6]. The band from 1850 cm^{-1} to 1650.23 cm^{-1} was due to carbonyl stretch of the esters and the stretch at 1381.55 cm^{-1} was due to carbon hydrogen stretch(-CH) of the polymer[2]

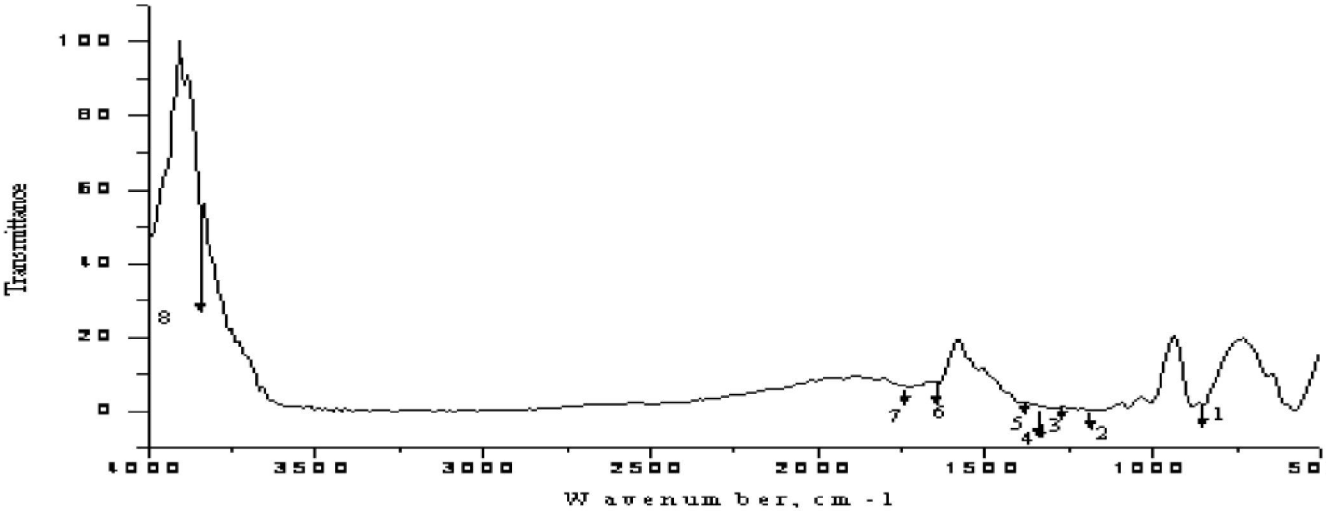

Figure 1 : FTIR spectrum of PHA from NB medium

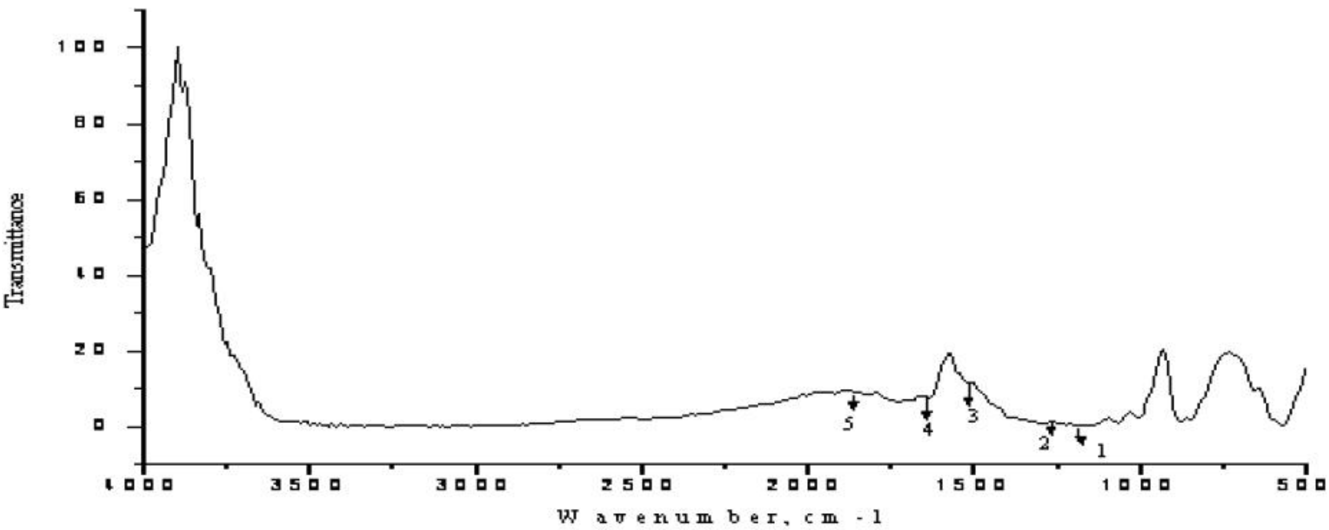

Figure 2 : FTIR spectrum of PHA from medium amended with Molasses as carbon source

PHA from medium amended with distillery effluent as carbon source

FTIR analysis of the isolated polymer from distillery effluent, revealed absorption bands at 1724cm^{-1} to 1728.70 cm^{-1} corresponding to the ester carbonyl group showing the presence of PHB[8] and the peak at 1281.64 cm^{-1} corresponds to the –CH group[11]. The absorption band at 1688.52cm^{-1} is found to be that of C=O valence vibration of PHA.

PHA from medium amended with whey as carbon source

FTIR spectrophotometer showed different spectrum in case of whey as carbon source. The peak at 3497.67-3700cm^{-1} was of that of hydroxyl stretching (-OH) of the polymer[6]. The band at 2925.59 cm^{-1} was found to be that of aliphatic stretching[3] of PHA. The band at 1245.78 cm^{-1} till 1319.21 cm^{-1} marks the Carbon oxygen carbon stretch of the polymer[4]. The peak at 1735.81 cm^{-1} and 1419.52 cm^{-1} was due to carbon oxygen stretching (C=O) of the esters and at 1375.22 cm^{-1} was due to the Carbon hydrogen(-CH) stretch of the aliphatic groups[2].

PHA from medium amended with coke industry waste as carbon source

The polymer from coke industrial waste showed absorption bands at 1727 cm^{-1}, which were corresponding to the ester carbonyl group[1]. The band at 2948.02 cm^{-1} was attributed to that of C–H stretching[3]. Specifically, the peaks at 1000–1125 cm^{-1} range and 1150 cm^{-1} region denote the presence of ester,

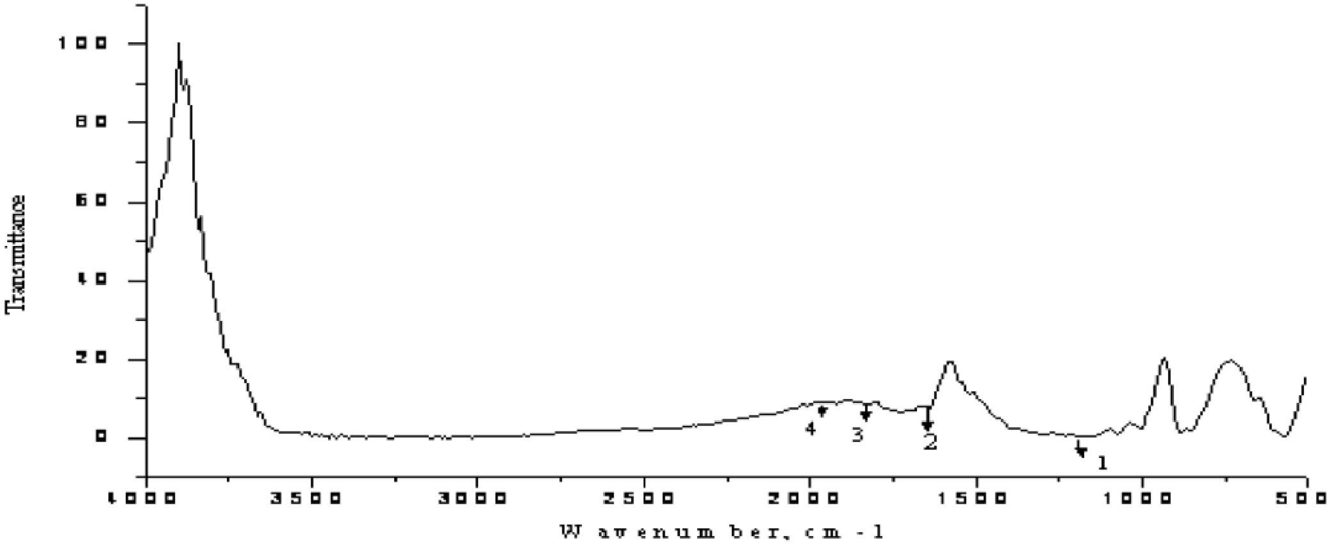

Figure 3 : FTIR spectrum of PHA from medium amended with Distillery effluent as carbon source

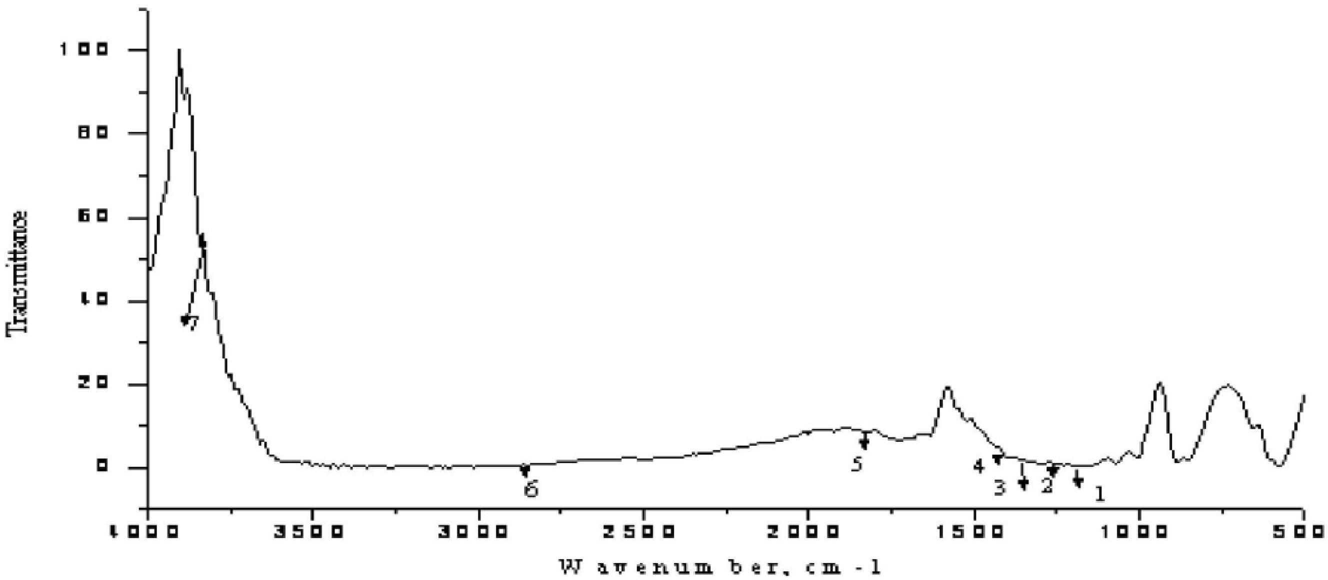

Figure 4 : FTIR spectrum of PHA from medium amended with Whey as carbon source

which are the characteristics of the polymer.

PHA from medium amended with glycerol as carbon source

FTIR spectra of the polymer showed prominent peaks at 1726.29cm^{-1} and 1276.88 cm^{-1} corresponding to the specific rotations around carbon atoms specific to certain functional groups of the polymer[11]. The peak at wavemnumber1726.29 cm^{-1} corresponds to C=O stretch present in the biopolymer. Thus, these peaks are corresponding to the peaks obtained for the standard PHA. The peaks at 2931.59 cm-1 and 3749.36 cm^{-1} corresponded to that of aliphatic –CH group and hydroxyl group stretch[4] and the 1281.64 cm^{-1} corresponding to the –CH group.

It was found that the PHA produced from different wastes as carbon sources had similar functional groups. The carbonyl group of the ester was prominent in almost all the spectra which ranged from 1700-1800 cm-1[11]. The region in and around 3400-3770 cm-1 was common in the PHA produced from NB, and the PHA from whey, coke industrial waste and glycerol from biodiesel industry and it can be attributed to that of hydroxyl stretching[6]. Thus the peaks of the different functional groups corroborates with the peak reported in the PHA obtained from the NB media[8].

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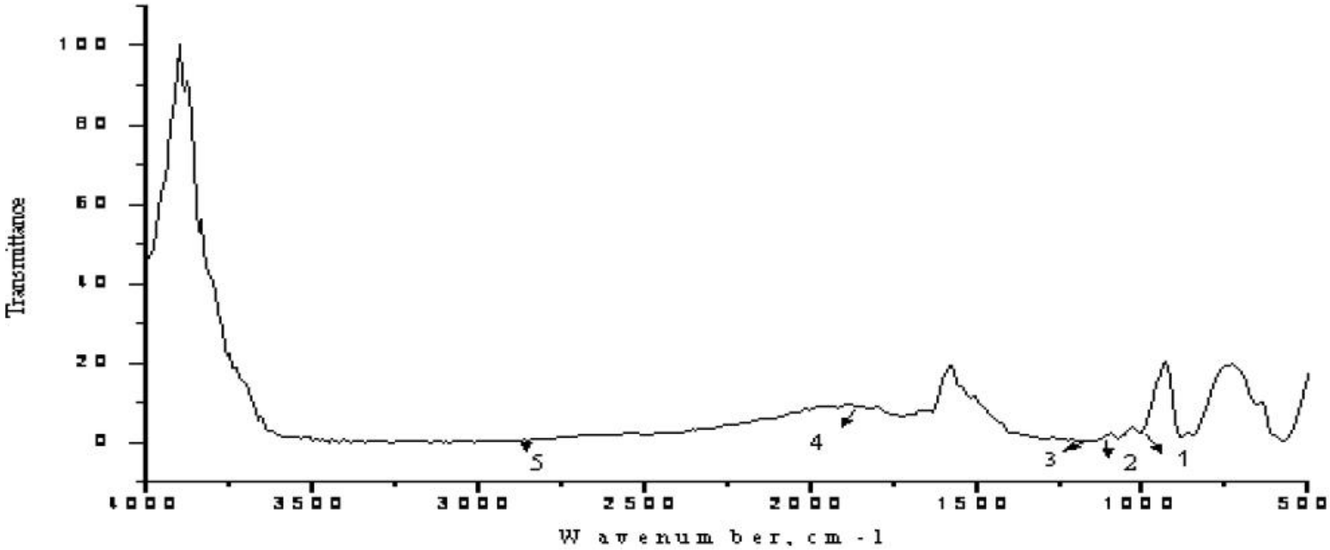

Figure 5 : FTIR spectrum of PHA from medium amended with Coke industry waste as carbon source

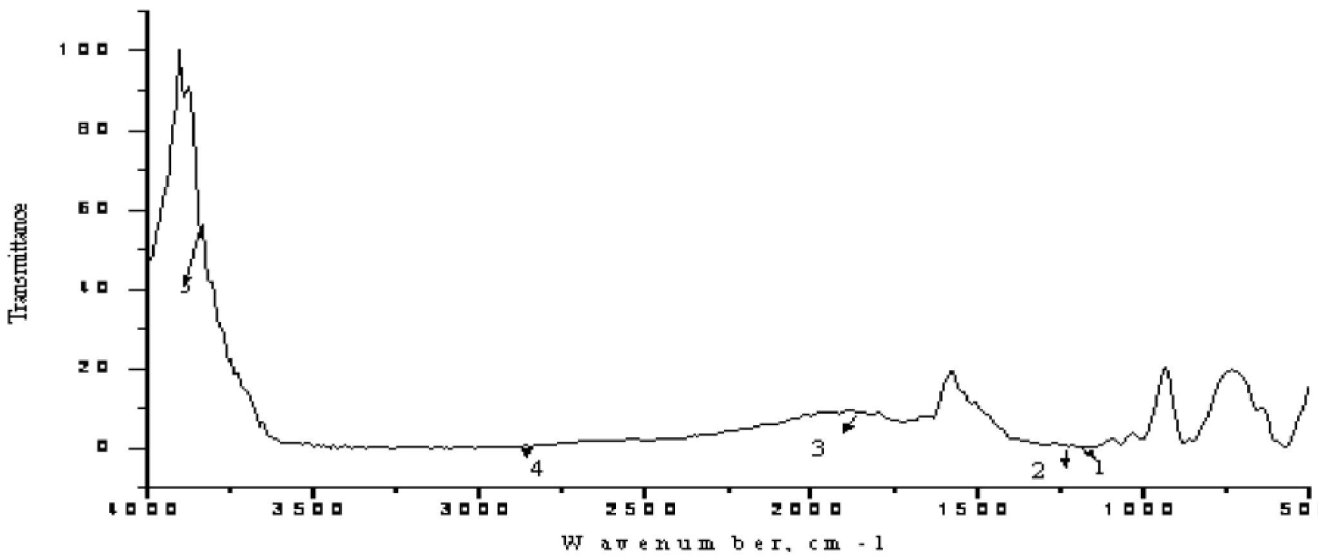

Figure 6 : FTIR spectrum of PHA from medium amended with Glycerol as carbon source

DISCUSSION

The production of PHA under structurally unrelated carbon sources were previously carried out by different researchers[11]. The IR spectrum of the PHA from various agricultural by products matched with that of the PHA produced from Nutrient medium, concluding that these agro industrial wastes can be used as feed for the microbes producing the biopolymer under appropriate conditions.

Within our country there are numerous agro based industries releasing the bio effluents which in turn disturbs the ecosystem. To avoid the environmental problems, these industries can be directed to utilize the effluents as carbon sources for the production of high quality biopolymers which has numerous applications in diverse fields. There are enormous applications of these biopolymers like packaging industry, medicine, pharmacy, and agriculture[8].

PHA can completely rule out the petroleum based non degradable polymers that are highly toxic. It can be easily degraded to H_2O and CO_2 under aerobic conditions and to CH_4 under anaerobic conditions by the action of micro organisms and it proves good as an environmentally friendly plastic or polymer. Thus by the present study it is possible to reduce the final production cost of the polymer suggesting its easy bulk production. Not only has the production of PHA, this study also suggested a new form of technology for the treatment of different industrial wastes.

CONCLUSION

Bacillus subtilis ANM1 is a soil bacterium efficiently synthesizing PHA using different nutrient sources. The cheapest of the industrial raw material is sugarcane molasses that had maximum production and the functional groups of the polymer from molasses also matched with that of the standard PHA. Thus cheap raw materials can be used for the production of high quality polymers for future perspective.

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