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OPTIMIZATION OF POLYHYDROXALKANOATE ACCUMULATION BY *KLEBSIELLA SP.* NCCP-138 ISOLATED FROM OIL CONTAMINATED SOIL

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ABSTRACT

Hydrocarbon polymers of microbial origins, such as polyhydroxyalkanoate (PHA) are considered to be major components of biodegradable plastics. PHA producing bacterium was isolated from oil contaminated soil and identified as *Klebsiella species* NCCP- 138 on morphological, cultural, biochemical tests and 16S rRNA sequence analysis. Maximum PHA accumulation of 1.89g/l was achieved with an inoculum size of 10% (0.8 OD_{530nm}) and the isolate was grown in PHA production medium containing 2.5% Mannitol, 0.5% Yeast Extract, 1% Peptone and pH 7.5 at 35°C under static condition for 72 hr. Further characterization of PHA was carried out using Fourier transform infrared spectroscopy (FT-IR).

KEY WORDS: PHA, *Klebsiella species* NCCP-138, Polyhydroxyalkanoate, Fourier transform infrared spectroscopy.

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INTRODUCTION

Modernization and progress has had its share of disadvantages and one of the main aspects of concern is the pollution, it causes to the earth – be it land, air, and water[1]. Even though plastic waste is a major environmental problem, it has become an integral part of contemporary life because of many desirable properties. The disadvantage of plastics is the difficulty in their disposal. This has prompted many countries to start developing biodegradable plastics[2-4]. Prokaryotic organisms, including gram positive and gram negative bacteria, are known to produce a class of storage lipids known as polyhydroxyalkanoates (PHA) that serve as endogenous carbon and energy source during starvation periods[5]. PHA is accumulated by a wide range of bacteria when a carbon source is provided in excess and one or more essential nutrient is limited[6-7]. PHA plays a significant role in the survival of the microorganisms under conditions of environmental stress such as osmotic pressure, desiccation and UV irradiation[8-9]. Polymers synthesized by microorganisms such as PHA are considered to be good substitutes for synthetic polymers, since they possess material properties similar to those of synthetic polymers currently in use[10].

PHAs are usually produced when carbon sources are in excess where it is assimilated, converted into hydroxyalkanoate (HA) compounds and finally polymerized into high molecular weight. PHAs which are stored as water insoluble granules in the cell cytoplasm[11]. The growth of bacteria and PHA accumulation is influenced by a variety of nutritional factors and physical factors. Nutritional factors include the availability of substrates and nutrients. Physical factors include pH, temperature and response to free molecular oxygen. The present study focuses on the use of novel bacterium *Klebsiella species* NCCP- 138 isolated from the soil, to accumulate PHA. To our knowledge, this is the first study on PHA accumulation using *Klebsiella species* NCCP- 138. Optimization of various physico- chemical parameters for PHA accumulation by *Klebsiella species* NCCP- 138 and characterization of PHA by FT-IR analysis are discussed in this paper.

MATERIALS AND METHODS

(i) Isolation, screening and identification

For screening of Polyhydroxyalkanoate (PHA) accumulating bacteria, oil contaminated soil sample was collected. Serial dilutions of the soil sample were prepared in sterile phosphate buffered saline (pH- 7.2). The aliquots were spread on sterile Nutrient Agar medium plates and incubated at 30°C for 24 hrs. Primary screening was performed for the presence of lipid granules by the Sudan Black B staining method[12]. Isolates exhibiting the presence of lipid granules were further screened for PHA accumulation by the Nile blue A plate assay method[13]. Colonies exhibiting fluorescence were scored as PHA accumulators. Identification of the promising isolate was done on the basis of morphological, cultural and biochemical test referring Bergey's Manual of Determinative Bacteriology[14] and the strain was confirmed by 16S rRNA analysis which was carried out at SciGenom Labs Pvt Ltd, Kerala, India.

(ii) Media and growth conditions

The isolate was cultivated in PHA production medium containing 1% yeast extract, 1% peptone, 0.1% Na_2HPO_4 and 0.02% $MgSO_4$ with 2% (w/v) glucose as carbon source[15]. The isolate was grown in 100 ml Erlenmeyer flask containing 20 ml of the medium. The flask was inoculated with 2.5% (v/v) overnight grown culture; and was incubated at 30°C for 72 hrs under static condition. All the experiments were carried out in triplicates.

(iii) Extraction & quantitative estimation of PHA by hypochlorite method:

The isolate was grown as mentioned earlier and the PHA was extracted from the isolate by using the Rapid Hypochlorite method[16], where the centrifuged cell pellet was suspended in 10 ml of 4% sodium hypochlorite solution and incubated at 30°C for 30 min. The PHA pellet obtained was washed with distilled water and acetone respectively. After washing, the pellet was dissolved in 5 ml of hot chloroform. The chloroform was allowed to evaporate by pouring the solution in glass Petri plate and placing it at 4°C. The PHA powder was collected for further analysis after evaporation. Quantitative estimation of the PHA extract was done by Slepecky and Law method[17]. The absorbance was measured (Systronics double beam spectrophotometer 2203) at 235 nm.

(iv) Effect of time on PHA accumulation

Time dependent studies were carried in triplicates by inoculating the culture in production medium. The medium was incubated at different time intervals ranging from 12 hrs to 96 hrs and the withdrawal of the sample was done after every 12 hrs.

(v) Effect of aeration on PHA accumulation

Effect of aeration on PHA accumulation was checked by incubating the flask inoculated with the isolate and incubating under static and shaker conditions at 30°C for 72 hrs.

(vi) Effect of different carbon sources on PHA accumulation

The effect of different carbon sources on PHA accumulation was done by substituting glucose in the PHA production medium with 2% (w/v) of the sugars like Sucrose, Fructose, Glycerol, Starch, Maltose, Mannitol and Galactose. PHA accumulation was also checked by using varying concentrations ranging from 0.5% to 4.0% of the optimized sugar.

(vii) Effect of varying concentrations of yeast extract and peptone on PHA accumulation

PHA accumulation was checked by using various concentrations of yeast extract and peptone ranging from 0.1% to 1% (w/v). Yeast extract (w/v) and peptone (w/v) were also used in combinations at varying concentrations of 0.5% yeast extract and 0.5% peptone, 0.5% yeast extract and 1% peptone, 1% yeast extract and 0.5% peptone and 1% yeast extract and 1% peptone.

(viii) Effect of temperatures and pH on PHA accumulation

Optimization of PHA accumulation by the isolate was investigated at various temperatures (0°C, 25°C, 35°C, 45°C and 55°C) and at different pH values ranging from pH 4.5 to 10.5, using PHA production media with 2.5% (w/v) mannitol, 0.5% (w/v) yeast extract and 0.5% (w/v) peptone.

(ix) Effect of inoculum size on PHA accumulation

For optimization of PHA accumulation initial inoculums of the isolate were added in range 5-25% (O.D 0.8 at 530nm) in the optimised PHA medium.

(x) Effect of concentration of sodium hypochlorite on PHA extraction

Also the optimum concentration of sodium hypochlorite required for the extraction of PHA was estimated[16]. Concentrations ranging from 1%- 4% (v/v) were used.

(xi) Characterization of PHA by FTIR

Powder of PHA extracted from the cells was also subjected to FT-IR analysis. For this, extracted PHA was dissolved in hot chloroform (AR) and the solvent was allowed to evaporate. After evaporation of the solvent, the powder was placed on KBR window. The IR spectrum of PHA was taken at 400–4000 cm^{-1} in a Shimadzu FT-IR[18].

RESULTS AND DISCUSSION

1. Screening, isolation and identification

From the oil contaminated soil 5 isolates were obtained on Nutrient Agar medium and were screened for the presence of lipid granules by Sudan black B staining[19] and Nile blue A plate assay method[13].

Only one of the isolates showed the presence of lipid granules and exhibited fluorescence. The isolate was scored as a PHA accumulator and was selected for further study. The isolate was identified as *Klebsiella species* based on cultural, morphological and biochemical tests and designated as *Klebsiella species* NCCP-138 (accession number NCBI- AB558497.1) using 16S rRNA sequence analysis. The amount of PHA accumulated in the isolate was found to be 0.25g/l which was extracted by rapid hypochlorite method and measured by Slepecky and Law method. The organisms that accumulate Polyhydroxyalkanoates (PHA) have been reported from various environments such as soil, mangrove swamps, sewage sludge, marine water and ponds[18, 20-22]. Contaminated environments contain essential nutrients and are enriched in conditions for PHA production. So these environments may have a large number of PHA producing bacterial strains[22].

2. Effect of time on PHA accumulation

The optimum time for PHA accumulation by *Klebsiella species* NCCP-138 was determined at the 72nd hr after which there was a decrease in the PHA accumulation. The accumulation of polymer begins in the late log phase of growth and the maxima achieved during the stationary phase of growth[16]. This time dependent reduction in polymer after 72 hrs may be due to lack of micronutrient as well as increase in bacterial metabolites that may have negative effect on the PHA production[23]. In case of *Ralstonia eutropha* NRRL B14690 maximum PHA concentration of 3.8 g/l was obtained after 60 hrs growth[24] whereas *Rhodobacter sphaeroides* N20 showed a PHA yield of 8.02 g/l after 96 hrs of growth[25]. Similarly PHA yields increase with time dependent manner in *Bacillus* sp. and highest yield 5.311 g/l was obtained after 72 hrs of growth[26]. It is therefore important to harvest cells at the optimum time to obtain a maximum yield of PHA[27].

3. Effect of aeration on PHA accumulation

Optimum accumulation of PHA was higher under static condition (0.31g/l) than shaker condition (0.22g/l). Small variations in oxygen availability, can lead to significant changes in the metabolite distribution of any bacterial cultures[28]. PHA accumulation was stimulated under oxygen limitation by *Ralstonia eutropha* and *Azotobacter beijerinckii*[29]. However, in *Pseudomonas* sp. K, the limitation of dissolved oxygen concentration decreased the rate of biomass growth and PHA accumulation[30].

4. Effect of various carbon sources on PHA accumulation

Amongst the various carbon sources, mannitol showed maximum PHA accumulation (0.44 g/l). For *Klebsiella species* NCCP-138 mannitol was substrate of choice, giving the highest yield of PHA, followed by glucose (Fig 1). PHA accumulation was checked at varying concentrations of mannitol and maximum of 0.75 g/l was obtained at 2.5% mannitol. With increase in mannitol concentration, PHA accumulation was seen to increase till a threshold, after which it declined (Fig 2). These reports were similar to the PHA accumulation in *Rhizobium meliloti* 14 which was most favoured by mannitol (PHA- 3.5 g/l) and sucrose (PHA- 2.14 g/l)[31] whereas *Vibrio* sp. BM-1 was able to utilize some of the other carbon sources (*i.e.*, starch, fructose and mannitol) as nutrients for the accumulation of PHA[32]. In most bacteria, cells synthesize PHA under growth-limiting substrates other than carbon sources such as nitrogen, phosphorus or oxygen[33]. Carbon sources serve three different functions within the organisms,

biomass synthesis, cell maintenance and PHA polymerization[27].

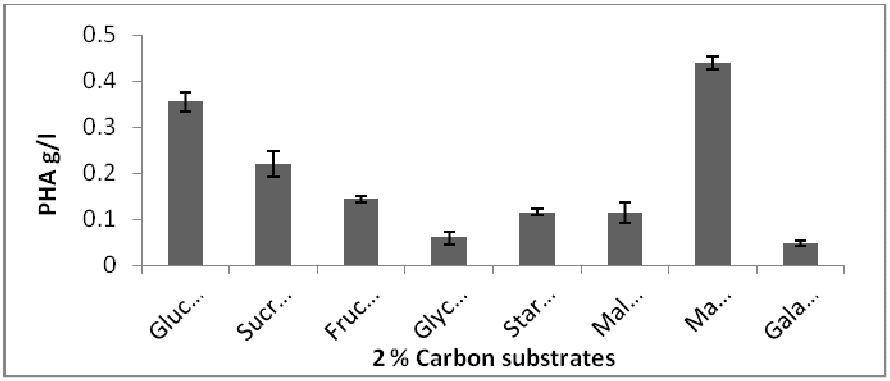

Figure 1
Effect of carbon sources on PHA accumulation

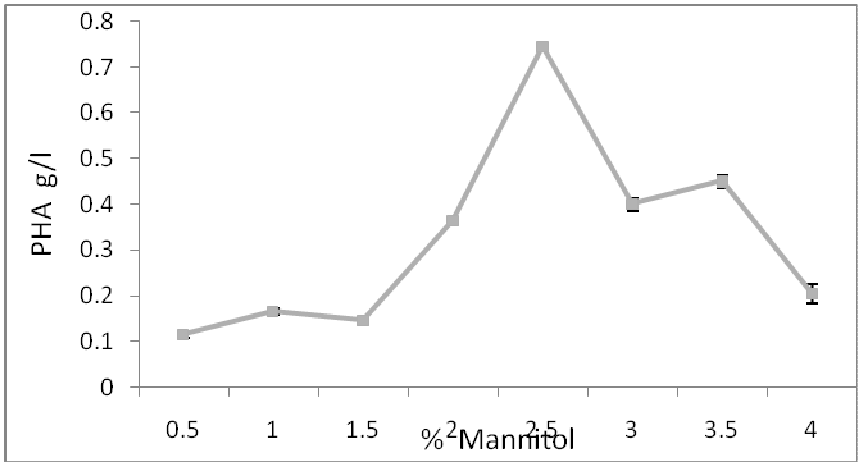

Figure 2
Effect of varying concentration of Mannitol on PHA accumulation

5. Effect of varying concentration of yeast extract and peptone on PHA accumulation

The amount of PHA accumulated by *Klebsiella species* NCCP- 138 with 2.5% mannitol was 0.96g/l at 0.5% yeast extract and 0.91g/l at 0.5% peptone (Fig 3). However, further increasing the yeast extract concentration did not elevate the PHA content. Theoretically, yeast extract positively affects the growth of microorganisms but a steady decline in PHA accumulation was observed after a threshold. The consumption of excessive yeast extract by microorganisms can cause them to grow rather than produce PHA[34]. The amount of PHA accumulation in an organism depends on the carbon to nitrogen ratio. The quantity of PHA accumulation increased as carbon to the nitrogen ratio increased[35]. The medium containing yeast extract increased cell concentration as well as PHA production by *Vibrio* sp. BM-1[32]. The PHA concentration reached its highest value of 1.065 g/l when 0.5% yeast extract and 1% peptone were used in combination (Fig 4). *Bacillus species* accumulated maximum PHA in carbon and nitrogen rich nutrient media (7.150 g/l) [26].

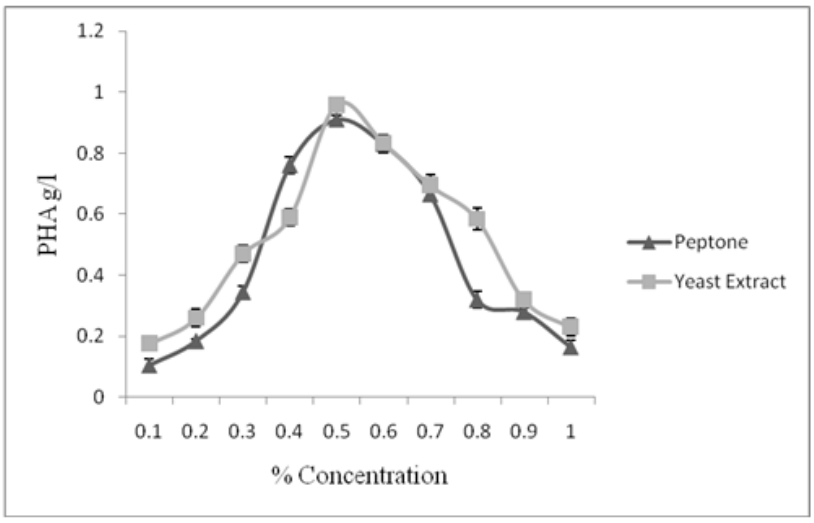

Figure 3
Effect of varying concentration of yeast extract and peptone on PHA accumulation

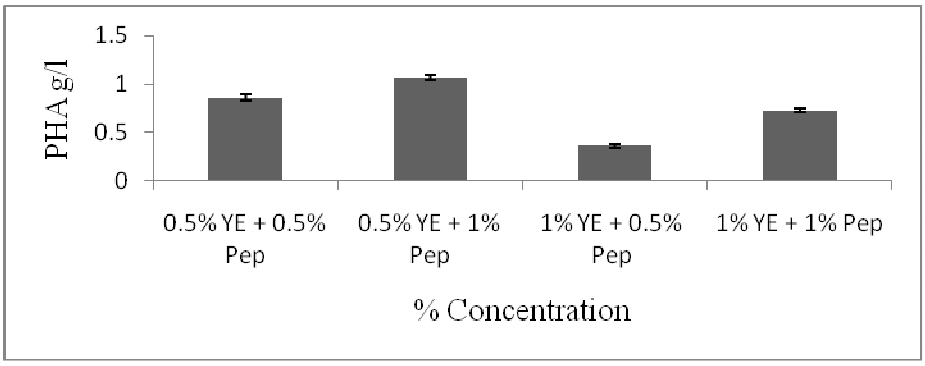

Figure 4
Effect of yeast extract (YE) and peptone (Pep) combination on PHA accumulation

6. Effect of temperature and pH on PHA accumulation

Maximum PHA accumulation 1.21 g/l was obtained at 35°C (Fig 5). The temperature of incubation plays a crucial role in the accumulation of PHA by the organisms. Temperature is a highly significant factor as proteins and enzymes begin to break down and lose functionality at certain temperatures[11]. *Rhodobacter sphaeroides* N20 also showed maximum PHA production (8.02 g/l) at 37 °C[25]. Maximum amount of PHA (1.37 g/l) was obtained at pH 7.5 (Fig 6). Similar reports are observed in *Rhizobium* strain grown on yeast extract mannitol broth at pH 7.0 was 0.01-0.5 g/l [36]. The pH value ranging from 6.0-7.5 is optimum for PHA production by *Alcaligens latus*[37]. The optimum growth of isolate VK-12 of genus *Burkholderia* was observed at pH 7.0 with maximum accumulation of PHA of 3.5 g/l[38]. The bacterial isolates belonging to different genus *Pseudomonas, Citrobacter and Enterobacter* showed highest percentage PHA production of 7.4, 6.9 and 5.9 g/l respectively at 37°C with cooking oil as carbon source at neutral pH after 72 hrs of incubation[22]. All the above data reveal that pH plays a major role in PHA accumulation and maximum PHA yield is obtained at neutral pH.

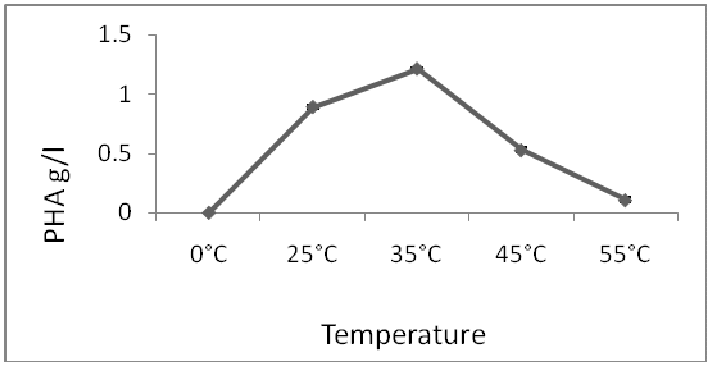

Figure 5
Effect of temperature on PHA accumulation

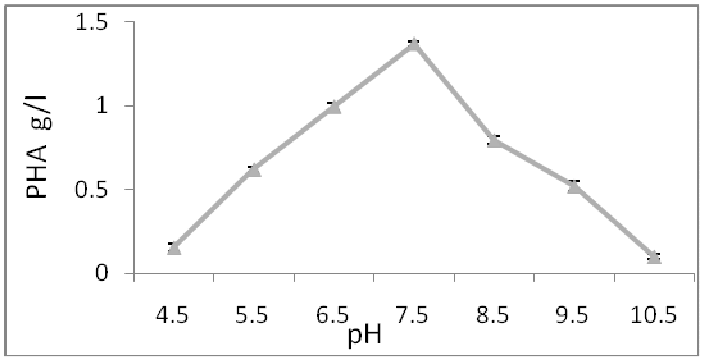

Figure 6
Effect of pH on PHA accumulation

7. Effect of initial inoculums on PHA accumulation

10% (v/v) of initial inoculum size of *Klebsiella species* NCCP- 138 was able to accumulate high amounts of PHA (1.55 g/l) (Fig 7). *Bacillus* spp was reported to require 10% inoculum to produce maximum PHA[39].

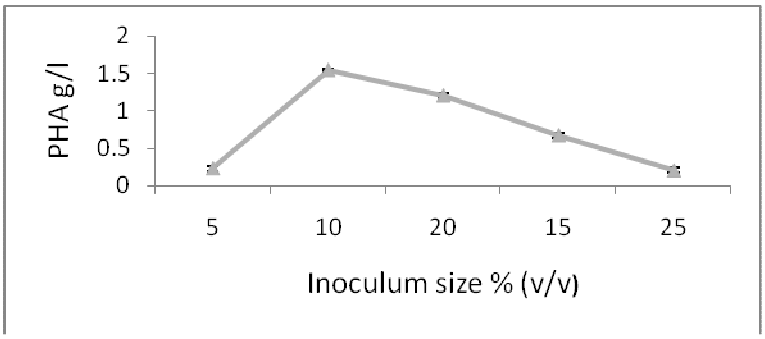

Figure 7
Effect of inoculum size on PHA accumulation

8. Effect of varying concentration of sodium hypochlorite for PHA extraction:

Maximum concentration of PHA (1.89 g/l) was extracted from the isolate, at a concentration of 3% hypochlorite (3% w/v of active chlorine) (Fig 8). Greater amounts of PHA could be extracted from *Vibrio* sp. 85/6 with the rapid hypochlorite method (2% w/v of active chlorine) which was simple and quick[16]. Prolonged contact with hypochlorite during extraction of PHA is reported to affect the polymer, leading to decreased molecular weight[40].

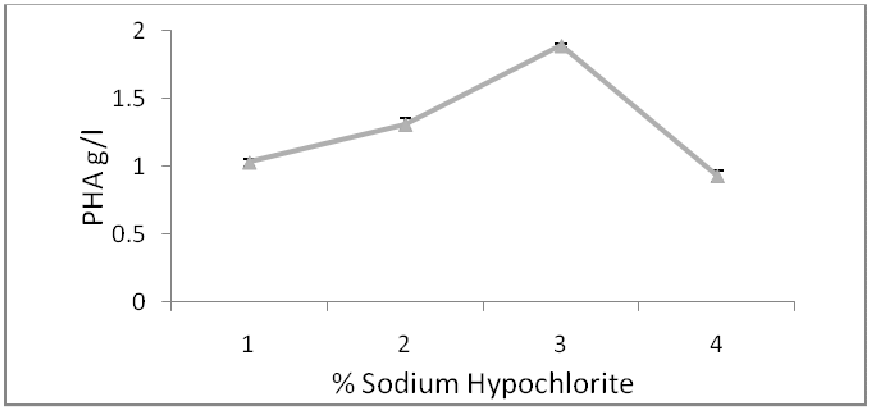

Figure 8

Effect of varying concentration of sodium hypochlorite (% w/v) on PHA accumulation

9. Characterization of PHA

FTIR spectrum of PHA extracted from *Klebsiella species* NCCP- 138 was recorded in the range of 4000-400 cm^{-1} and spectroscopic analysis showed the presence of broad bands responding to the groups CH, C=O and C-O indicating the structure similar to PHA. The peak values obtained in this study coincides with previous results of Kumar and Prabakaran[18]. Also a marked peak for the ester carbonyl bond was observed at 1650 cm^{-1} (Fig 9). A similar peak for the ester carbonyl bond was observed for PHA in *Saccharophagus degradans*[41] and *Alkaligenes eutrophus*[42].

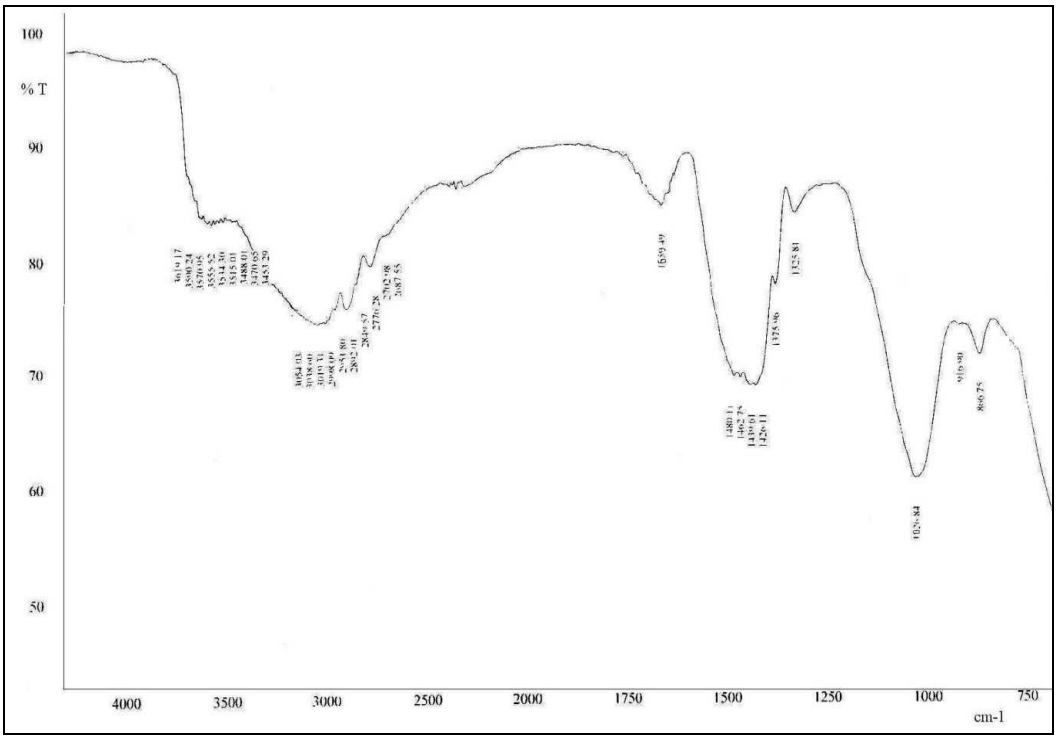

Figure 9
FTIR spectrum obtained for PHA extracted from Klebsiella sp. NCCP-138

CONCLUSION

Klebsiella species NCCP- 138 isolated from oil contaminated soil is capable of producing PHA biopolymer. The yield of accumulated PHA was optimized for various physico-chemical parameters and the presence of the PHA was confirmed by the FT-IR analysis. PHA production by this organism has not yet been reported and hence accumulation of PHA in *Klebsiella species* NCCP- 138 is novel. A polymer can be accepted commercially only if it possesses the necessary physical, chemical and mechanical properties similar to the petrochemical plastics. Further studies shall be carried out to determine the mechanical properties of the extracted PHA in order to check their compatibility with petrochemical plastics. In order to produce PHA economically, the use of cheap and easily available raw materials is desirable which needs to be analyzed further.

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