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ISOLATION, OPTIMIZATION AND PRODUCTION OF POLYHYDROXYALKANOATES (PHA) FROM SOIL BACTERIA

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ABSTRACT
Petroleum derivatives such as plastics have become fundamental elements of our day-today life. This is due to the desirable properties such as durability and strength. The uncontrolled utilisation of the synthetic plastic materials by human beings leads to hazardous environmental pollution because of improper disposal measures. Thus, the biodegradable polymer, PHA, produced from various microorganisms is a good alternative for the petroleum derived plastics. Polyhydroxyalkanoates occur as an insoluble cytoplasmic inclusion in many Eubacteria and also in halophilic Archaebacteria, as a storage component for carbon and energy. The most important benefit of these biodegradable polymers is that they are totally degraded into components like water, carbon dioxide and methane by many microorganisms under anaerobic conditions. Hence, their disposal does not cause any harmful effect to the environment. They also have a wide range of applications because of their biocompatibility, biodegradability and negligible toxicity to cells. Their application includes, packaging of bottle manufacture, and as a coating material. It has been used in medical fields for the manufacture of non woven materials, polymer films, sutures and pharmaceutical products that are used in surgery, transplantation and tissue engineering. Recent study shows that they can also be used for the production of biofuels. Thus, the present study focuses on the isolation and identification of soil bacteria capable of producing polyhydroxyalkanoates. The bacterial isolate *Bacillus* sp. was found to possess a higher level of PHA production in the presence of an inexpensive substrate (rice chaff). However, the isolate *Azotobacter* sp., showed lower levels of PHA production when compared with the literature. The percentage of PHA accumulated by *Bacillus sp.* was found to be 51.49% .

KEYWORDS: Polyhydroxyalkanoates, inclusions, bioplastic.

INTRODUCTION

Pollution may lead to critical problems in the global geochemical cycles as well as the sustainable habitation of humans and other organisms. Various types of hazardous substances can enter the natural environment by a number of natural and/or anthropogenic activities, disturbing the living systems along with many adverse changes in the environment. In different urban areas huge mega lexes have been constructed which are not sustainable and they experience problems with waste management, heat islands, increasing pollution and crowding of increasing population *etc*. CO_2 is toxic for pregnant women and when exposed, the foetus may be harmed. Likewise, car exhaust gases damage health of both adults and children, leading to change in behaviour and psycho-social development of children (Chelala, 2010; Markert *et al*., 2011).

Plastic is any synthetic or semi-synthetic organic polymer. They are of two types, thermoplastics and thermosetting polymers. Thermosetting polymers, also known as thermosets, solidify into a permanent shape. They are amorphous and considered to have infinite molecular weight. Thermoplastics, on the other hand, can be heated and remoulded over and over again. They are designed in a way that it has a long life span therefore, causing them to be inert to chemical and natural breakdown. Thus, when these plastics are disposed into the environment, they cause pollution due to its inertness. As the natural environment is continuously polluted by these hazardous plastics, the development and production of environmental- conserved biodegradable plastics becomes necessary in order to reduce our usage of synthetic plastics. Thus, bio-based materials such as polynucle-otides, polyamides, polysaccharides, polythioesters, polyoxoesters *etc.* can be used as an alternative for synthetic plastics. Among these polyhydroxyalkanoates (PHA's) which belongs to the group of polyoxoesters are extensively used because of its biodegradability.PHA is the only bio-based polymer that can follow a closed loop bio-based-to-biodegradable life cycle. PHA is a family of naturally-occurring biopolyesters synthesized by various microorganisms. Lemoigne, in 1926, was the first to report the occurrence of PHA in *Bacillus megaterium* and the first patent about its potential application as plastic material was filed in 1962 (Patent number 30036959, James Noel Baptist, May 1962), but until 1982 they were not industrially produced.

A PHA molecule is typically made up of 600 to 35,000 (R)-hydroxy fatty acid monomer units. Each monomer unit harbors a side chain R group which is usually a saturated alkyl group but can also take the form of unsaturated alkyl groups, branched alkyl groups, and substituted alkyl groups although these forms are less common. Depending on the total number of carbon atoms within a polyhydroxyalkanoate monomer, they can be classified as either short-chain length PHA (scl-PHA; 3 to 5 carbon atoms), medium-chain length PHA (mcl-PHA; 6 to 14 carbon atoms), or long-chain length PHA (lcl-PHA; 15 or more carbon atoms). Polyhydroxyalkanoate accumulates as discrete granules to levels as high as 90% of cell dry weight in response to environmental stress and

nutrient imbalance (i.e. nitrogen and phosphorus limiting conditions). These inclusions are typically 0.2-0.5 µm size surrounded by phospholipid monolayer membrane (Getachew, 2016).

The key enzymes of PHA biosynthesis are the PHA synthases that catalyze the formation of the PHA chain. Various metabolic routes are realized towards the synthesis of the activate PHA precursor, (*R*)-3-hydroxyacyl-coenzymeA (CoA), which serves as substrate for the PHA synthase. The biosynthesis of PHB begins with the condensation of two acetyl-CoA molecules catalyzed by the β–ketothiolase (PhaA) resulting in the formation of acetoacetyl-CoA which is then reduced to (*R*)-3-hydroxybutyryl-CoA by the (*R*)-specific aceto-acetyl-CoA reductase (PhaB). (*R*)-3-Hydroxybutyryl-CoA is the activated precursor of PHB and substrate for the PHA synthase (PhaC). The genes encoding PHB biosynthesis proteins are often co-localized and organized in an operon. However, PHAs composed of mcl-(*R*)-3-hydroxyfatty acids are synthesized by diverting intermediates of fatty acid metabolism to (*R*)-3-hydroxyacyl-CoA and thus towards MCL-PHA. If the carbon source is oxidized to acetyl-CoA, excluding its formation by the fatty acid by β-oxidation pathway, then intermediates of fatty acid by *de novo* biosynthesis become the precursors for mcl-PHA biosynthesis and whose conversion is catalysed by the transacylase PhaG.

PHA degradation in natural environments such as soil, sea water and lake water has been evaluated. A number of microorganisms such as bacteria and fungi have the ability to excrete extracellular PHA-degrading enzymes to hydrolyse PHA and utilize it as carbon and energy source. Biodegradation was found to be dependent on a variety of factors such as microbial activity of the environment, the exposed surface area, temperature or pH, polymer composition and crystalline nature. PHA has attracted much attention due to its biodegradability and the possibility of using renewable resources for its synthesis. Due to its thermoplastic properties, it is considered as a promising environmentally friendly alternative to petrochemical plastics.

Many different applications have been described for bioplastics since the first industrial production of Biopol1 by ICI Ltd in 1982. PHAs behave as thermoplastics and thus, make them suitable for injection moulding, film applications, extruded sheet production and thermoforming. PHA is an appropriate material for packing because of its lightness and transparency. Moreover, it presents lower permeability to oxygen than conventional plastics which means better preservative properties for food. Like nylons, PHA can be processed into fibres, Chiral intermediates for chemical synthesis and for the synthesis of pharmaceutical products such as sutures. Hydroxyalkanoate methyl esters can be used as biofuels and are easily obtained from the esterification of PHA.

Thus, in this study, several strains of PHA accumulating bacteria were isolated from soil, characterized for their morphological properties. In addition, comparison of PHA production by the selected strain was done by growing bacterial cultures in Minimal Salt Medium containing inexpensive substrates such as Rice bran. Later, the absorbance of the extracted polymer was evaluated using UV-Vis Spectrophotometer and the purity of the extracted polymer was identified using FTIR Spectroscopy.

MATERIALS & METHODS

Three soil samples were collected from different places (garden, agricultural and termite soils). Isolation of bacterial strain was carried out by serial dilution of 1g of soil sample in 9ml of 0.85% sterile saline solution followed by plating 1ml of sample from dilution 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} onto sterile nutrient agar medium by pour plate technique. The plates were incubated at 37°C for 24 –48 hours. The bacterial isolates were subjected to various preliminary and biochemical tests for the purpose of identification. They were further compared for the production of PHA in shake flasks utilizing rice chaff as the substrate. The turbidity in the medium indicates the growth of the isolate.

The isolated strains were inoculated into the production medium (Mineral Salt Medium containing rice chaff as the carbon source (inexpensive substrate)). The pH of the media was maintained at 7. The culture flask was incubated in shaker at 150 rpm at 35°C for two days (Preethi *et al.*, 2012). 10ml of bacterial cell suspension was taken in a centrifuged tube. It was centrifuged at 6000rpm for 10 minutes. The supernatant was discarded. The pellet was washed with 10ml of saline. It was centrifuged again. To the pellet, 5ml of sodium hypochlorite was added. It was incubated at 37 for 10 minutes in a shaker. It was then, centrifuged at 8000rpm for 20 minutes.

The pellet was washed with diethyl ether. It was again centrifuged (6000 rpm for 10 minutes) and the pellet was assayed (Rawte and Mavinkuruve, 2002). The dry cell weight (DCW) was estimated in units of g/L (Du *et al*., 2001). Residual biomass was estimated as the difference between dry cell weight and dry weight of extracted PHA (Zakaria *et al*., 2010). This was calculated to determine the cellular weight and accumulation other than PHAs. The percentage of intracellular PHA accumulation is estimated as the percentage composition of PHA present in the dry cell weight.

Residual biomass (g/L) = DCW (g/L) - Dry weight of extracted PHA (g/L)

PHA accumulation (%) = [Dry weight of extracted PHA (g/L) / DCW (g/L)] × 100%

The PHA extracted by above methods was assayed by Slepecky and Law's method. PHA sample was treated with 4.5 ml of concentrated H_2SO_4 and placed in a boiling water bath for 10 minutes. On cooling absorbance was noted at 235 nm on UV-VIS Spectrophotometer. The extracted PHA samples were added with KBr and then evaporated. The depositors were then dried and an IR spectrum was recorded from 400 to 4000cm^{-1} range.

RESULTS & DISCUSSION

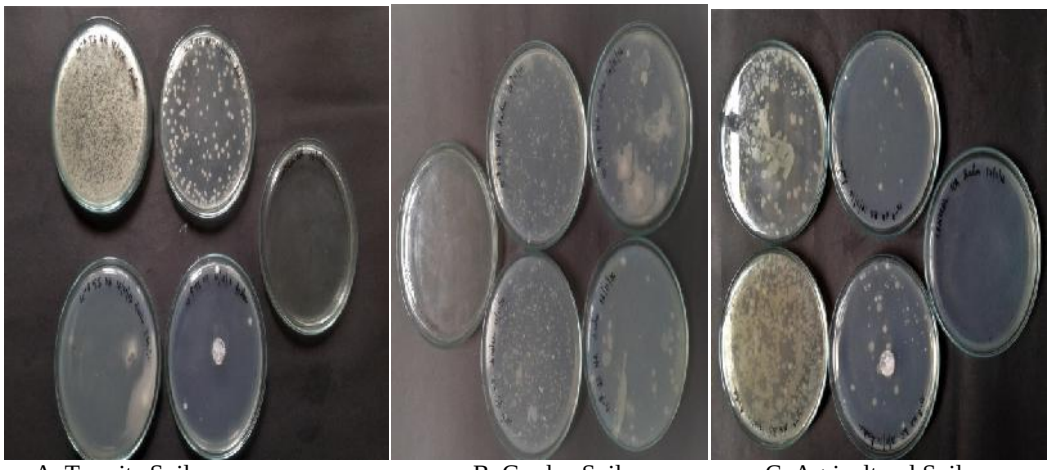
Twelve strains were isolated from the 3 soil samples by the pour plate method and identified by various tests (both microscopic and macroscopic) (Fig 1). 2 of the 12 isolates were identified as *Bacillus sp. and Azotobacter sp*. Since, the spores of *Bacillus* sp. is ubiquitous in nature, it was one of the isolate. Furthermore, from the literature it could be inferred that *Bacillus* sp., is an efficient PHA producer. Also, *Azotobacter* sp., was one among the isolates from soil which has shown to be an efficient PHA producer in

the literature. Generally, *Azotobacter* sp., is a good nitrogen fixer (Santimano *et al.,* 2009). Thus, this study was carried out with these 2 isolates in order to identify the efficacy of these strains from the species to produce PHA for bio-plastic production. The two selected strains, *Bacillus sp. and Azotobacter sp.* were grown in a Mineral Salt Medium containing rice chaff as the carbon source (inexpensive substrate). The growth was determined by the turbidity in the medium (Fig. 2).

The intracellular PHA was extracted by the rapid Sodium hypochlorite method. The extracted PHA was pale white or ivory coloured (Fig 3). Further, in order to determine which of the 2 isolates, an efficient PHA producer was, the extracted polymer was quantified. Quantitative estimation of the polymer revealed that *Bacillus* sp., could accumulate unequivocally about 51.49% of dry cell weight as PHA within 24 hours using the carbon substrate, Rice chaff. Generally, members of the genus *Bacillus* are known to accumulate PHA content ranging from 6.53 to 48.2% (Shamala *et al.,* 2003; Aslim *et al.,* 2002). But in this study the quantity was quite higher. Although, *Azotobacter* sp. had shown to be an efficient PHA accumulator in the literature, accumulating up to 64.02% of the cell, in the present study it showed only minimal PHA accumulation (34.78%). Thus, it can be inferred that the isolated strain of *Bacillus* sp., is an efficient PHA accumulator than *Azotobacter* sp. (Table-1.). The absorbance pattern of the extracted polymer was studied from 200-800 nm in UV-Visible Spectrophotometer. Presence of a peak between 230-240nm indicates the presence of ester group thus, confirming the presence of PHA molecule (Fig 4). FTIR spectroscopy of the extracted polymer indicated the presence of an intense band at 1726.12cm^{-1} which corresponds to the presence of aliphatic carbonyl group (C=O) group of R-CO-A in PHA polymer and the band at about 1058.12 cm^{-1} characterizes the valence vibration of the carboxylic group. (Fig 5).The peak at 1177.12 cm^{-1} confirmed the carbonyl group (C-O) stretching of the esters present in PHA. Apart from these, the polymer also showed a sharp peak at 1400.49 cm^{-1} corresponding to alkane (CH_2) groups and the band at 1636.8 cm^{-1} is the characteristic feature of the (C=O) of amide group, which absorbs even at lower frequency. These recordings indicate the presence of PHA polymer.

TABLE-1. Quantification Of Extracted Polyhydroxyalkanoates (PHA)

S.No	Strain Name	PHA	Dry Weight of PHA(g/ml)	Dry Cell Weight ((g/ml))	Residual Biomass ((g/ml))	% of PHA Accumulation ((g/ml))
1.	*Bacillus* sp.	PHA1	0.052	0.101	0.049	51.49
2.	*Azotobacter sp.*	PHA2	0.008	0.023	0.014	34.78



A. Termite Soil B. Garden Soil C. Agricultural Soil

FIGURE 1. Nutrient agar plate showing bacterial colonies isolated from termite soil sample

FIGURE 2: Production of PHA in shaker flask

FIGURE 3: Extracted PHA granules of *Bacillus* by Rapid Sodium hypochlorite method

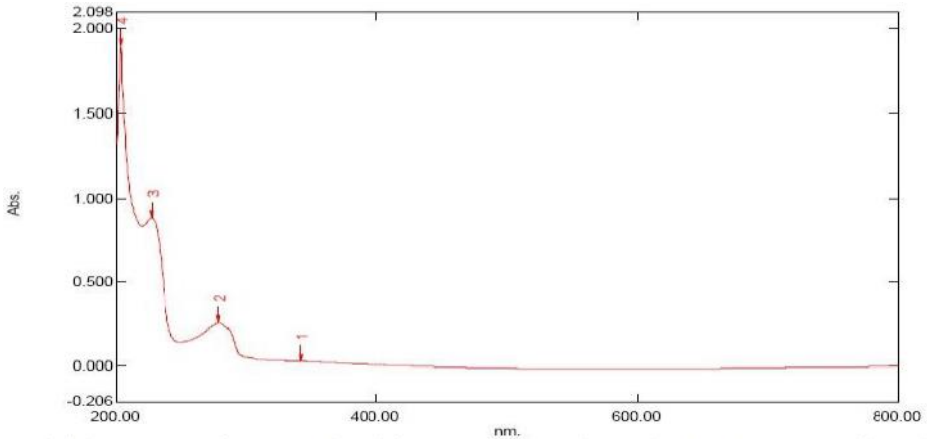


FIGURE 4: UV visible spectrum of PHA produced from *Bacillus* sp. The peak at 230-240 nm confirms the presence of ester group in the polymer

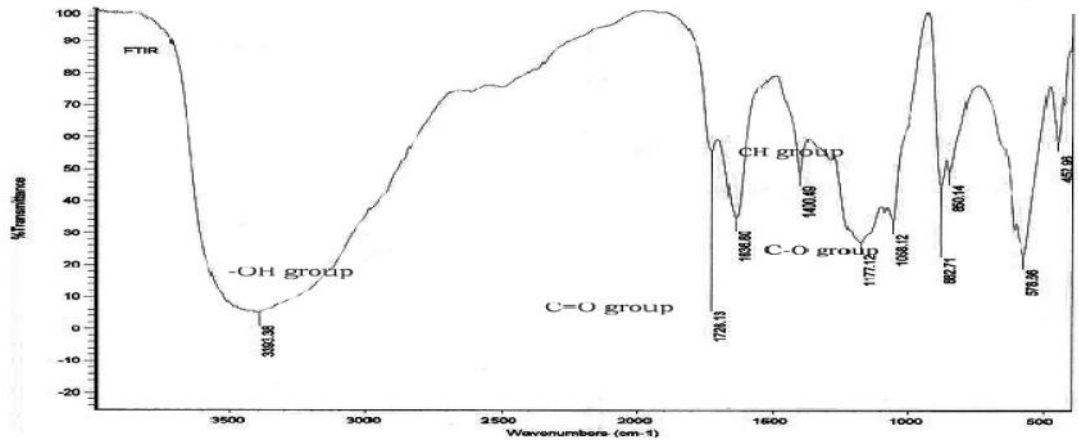


FIGURE 5. FTIR spectrum of PHA produced from *Bacillus* sp. indicates the presence of several functional groups in the polymer.

CONCLUSION

The high cost of polymer production is the major constrain in the commercialization of biodegradable plastics. For economical PHA production, cheaper renewable resources used as carbon feedstock and bacterial strains able to produce large quantities of intracellular PHA using such low-cost substrates are required. Wastes generated in the agricultural sector are abundantly available. These wastes are abundantly used as cattle feed since they have little economical value. These renewable agricultural residues are rich in carbohydrates. Members of the genus *Bacillus* sp. have innate ability to utilize diverse and cheap carbon wastes as they possess heterologous enzyme system capable of metabolizing the complex residues. Therefore, *Bacillus* sp. is now being explored industrially using agro-industrial wastes for economic PHA production. From this study it was determined that the isolated strain of *Bacillus* sp. can efficiently produce higher quantities of PHA with less fermentation time and less substrate concentration and with inexpensive substrate, as compared to the other reported organism and therefore, a potential strain to be explored for commercial PHA production.

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