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RESEARCH ARTICLE

PRODUCTION OF BIOPLASTICS FROM MICROORGANISMS.

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Abstract

The deleterious effects of synthetic plastics and their products have become a major concern for researchers. Bioplastics or plastics produced by the microorganism is a promising replacement for the conventional synthetic plastics. Polyhydroxyalkanoate a biologically produced biodegradable substance that has characteristic properties similar to that of conventional plastics. Polyhydroxyalkanoates are secondary metabolites of microorganisms which are produced under stressful conditions. In this work, four different samples were collected. These strains were then morphologically and biochemically characterized. The strains producing polyhydroxyalkanoates from each sample were identified by Sudan Black staining. A 48-hour culture of these strains was harvested and alkali lysis method was used to isolate polyhydroxyalkanoate and polyhydroxyalkanoate was quantified. Sample 2 had the highest polyhydroxyalkanoate accumulation % (95.65%). The method used for the production and isolation of polyhydroxyalkanoate was cost effective and ecofriendly .

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Introduction:-

Synthetic plastics are one of the greatest inventions of mankind and have been developed into a major industry and have become an essential part of our day to day life. They are designed in such a way that they are suitable for constant and long lasting performance causing them to be inert to natural and chemical breakdown. The durability of the disposed plastic had caused many serious environmental problems.

As dependence on synthetic plastics and their endless products have resulted in waste accumulation and greenhouse gas emission, recent technologies are more focusing on developing a bio-green substituent for plastic that exerts negligible side effects on the environment.

Polyhydroxyalkanoate (PHA), a biologically produced biodegradable substance which has similar characteristics of plastic have become a main focus for the research in finding a substituent for plastic. The PHA is the only bioplastics completely synthesised by the microorganism. PHA is the linear polyesters that are produced by bacterial fermentation of sugars or lipids which can be converted into CO_2 by microorganisms. They can be either thermoplastics or elastoplastic with the melting point ranging from 50 - 180 ◦C.

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PHA production is increased by the excess of carbon source and limiting the nutrients like nitrogen, phosphorous, sulphur, magnesium, iron etc (minimal media). It is secondary metabolite produced under stressful conditions. PHA is typically produced as a polymer of 103-104 monomers, which accumulates as inclusions of 0.2-0.5 micrometer in diameter.

PHA have rich properties depending on the structures over 150 different PHA monomers are reported homopolymers, random copolymers and block copolymers of PHA can be produced depending on bacterial species. PHA is thermoplastics, biodegradable, biocompatible, optically active and non-permeable.

As PHA can be used the field of packaging, medicine and much more, and due to cost-effectiveness & environmentally friendly properties, it is the promising alternative to the conventional plastics.

Materials And Methods:-

Materials

Samples, all the reagents used were obtained from SDFCL(Indian), Ranbaxy(Indian) and Fine Chemicals Ltd(Indian)

Sampling

Four samples were collected from different places to isolate the PHA-producing bacteria.

Table 1:- samples

Sample – 1	Sludge (Chamrajpet)
Sample -2	Sewage (Jnana Bharathi)
Sample – 3	Yeast Granules
Sample -4	Forest soil (Wayanad)

Isolation of Bacterial Strains:-

Isolation of bacterial strains was carried out by serial dilution of the samples in saline solution followed by plating of the samples onto nutrient agar media. The samples were then incubated at 37◦C for 48 hours.

Colony Characteristics:-

Once a bacterium has been obtained in pure culture, it has to be identified in order to study them [13].

Gram Staining:-

Gram staining [2, 4, & 9] is one of the techniques employed to analyse the bacteria and to classify them in order to study them.

Biochemical Test:-

For the biochemical characterization of the isolates biochemical tests [6, 10, 15, 17, 20, 21, & 22] were done for each isolate.

Motility of the bacteria was observed by hanging drop slide [11, 12, & 19]. This experiment is helpful in observing both motility and general shape of living bacteria.

Screening of Bacterial Strains for PHA Accumulation by Sudan Black Staining.

Bacterial strains were screened for the accumulation of PHA by Sudan black staining [3 & 18]. Sudan Black stain is a dye that is soluble in fat and insoluble in water and thus accumulates in fat globules of the cell. It is used for staining neutral lipids and triglycerides and some lipoproteins. Thus Sudan Black stain is used to identify PHA granules as they are fat globules.

Culturing of Micro-organisms in Minimal Media:

As PHA is a secondary metabolite and is produced under stressful conditions, it is very much necessary to culture the microorganisms in a minimal nutrient media [1, 7, & 24]. Minimal media is an unbalanced culture media which contain an excess of carbon sources but a very limited amount of oxygen, nitrogen, phosphorous, sulphur, or magnesium. This minimal media will provide the suitable conditions for active PHA production by the cells.

The bacterial strains identified to produce PHA by Sudan black staining were inoculated into the minimal nutrient media. 15 ml of each sample was directly inoculated into 50 ml of minimal media. Whereas in the case of yeast two granules (0.5g each) of dry yeast were inoculated. The samples were incubated for 48 hours.

Examination of Samples by Spectroscopy:-

The optical density of PHA produced by each sample was obtained by spectroscopy [16]. UV – Visible spectroscopy is an important technique to determine the formation and stability of PHA.

Extraction of PHA

The cells were harvested from a 48-hour culture by filtering using Whatmann filter paper. The filtrate was allowed to dry in the Petri plates overnight in the incubator. The dry cell mass was later scraped and weighed. 0.25 mg of all the samples were further used for the extraction of PHA. We used the method of alkali hydrolysis [14] for the extraction of PHA.

Dried biomass of 0.25 mg was suspended in 1.25 ml of water and pH was set (between 8 to 11) using 25% v/v ammonia solution. They were then incubated for 10 minutes at 50°C. It was further centrifuged at 6000 rpm for 10 minutes and washed with acetone. It was further dissolved in chloroform and filtered. The chloroform layer containing PHA was evaporated and dried overnight.

Quantification of PHA

It is very important to know the quantity of PHA in the different samples collected [7 & 25]. The bacterial culture was centrifuged at 6000 rpm to obtain the cell pellet and dried to estimate the dry cell weight (DCW) in units of g/L. Residual biomass was estimated as the difference between dry cell weight and dry weight of PHA extracted. This was calculated to determine the cellular weight and accumulation other than PHA's. The percentage of intercellular 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.

$$\text{PHA accumulation (\%)} = \frac{\text{dry weight of extracted PHA g/L}}{[\text{DCW g/L}]} \times 100$$

After 48 hours incubation at 37°C. The culture was collected and centrifuged at 10,000 rpm for 15 minutes and lyophilized.

The addition of methanol and water and vortexing the pellet and then centrifugation at 10,000 rpm for 15 minutes. Methanol is added to lyse the cells and to dissolve PHA. Water is added for separation of PHA in lipid solvent completely.

Results:-

Colony Characteristics:-

Bacterial colonies were characterised by colony Gram staining, characterization and biochemical tests. (Table 2 & table 3)

Table 2:- streak plate colony characteristics after 24 hours of incubation.

Sample number	Form	Margin	Elevation	Color	Light transmission
1	round	Smooth	convex	White	Translucent
2	irregular	Wavy	flat	White	Transparent
3	round	Smooth	Convex	Creamy	Opaque
4	irregular	Wavy	umbonate	White	Opaque

Table 3: Gram staining & Biochemical characteristics

Biochemical Test	Sample1	Sample2	Sample 3	Sample4A
Gram staining	-	+	-	-
Shape	Cocci	Bacilli	Cocci	Cocci
Motility	Motile	Non-motile	Non-motile	Motile
Fermentation Tests				
Glucose	A, G	A, G	A	A
Lactose	A, G	A	A	A
Sucrose	A, G	A	A	A
IMVIC TESTS				
Indole	+	+	+	+
MR	-	-		+
VP	+	+		-
Citrate utilization	+	+		+
Starch hydrolysis	-	-	+	+
Gelatin liquefaction	-	-	+	+
Nitrate reduction	-	-	_	-
Tryptone test	+	+	_	+
Mac Conkey Agar	-	-	_	+
Oxidase Test	+	+	+	+
Catalase Test	+	+	+	+

Examination of Samples by Spectroscopy, Extraction and Quantification of PHA

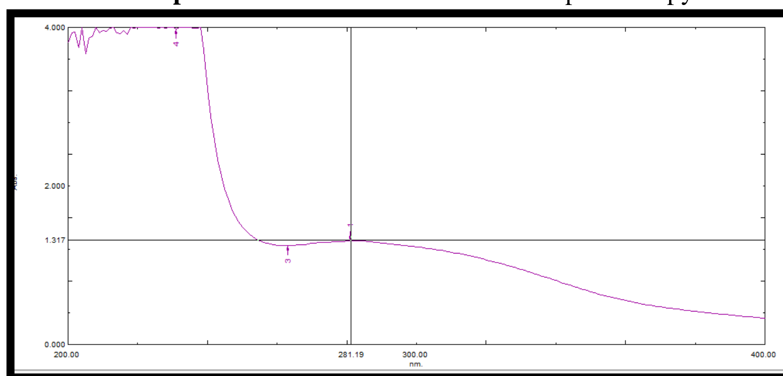
Optical density of PHA granules obtained from each sample was obtained using spectroscopy.

Table 4:- Optical density and absorption maxima of samples

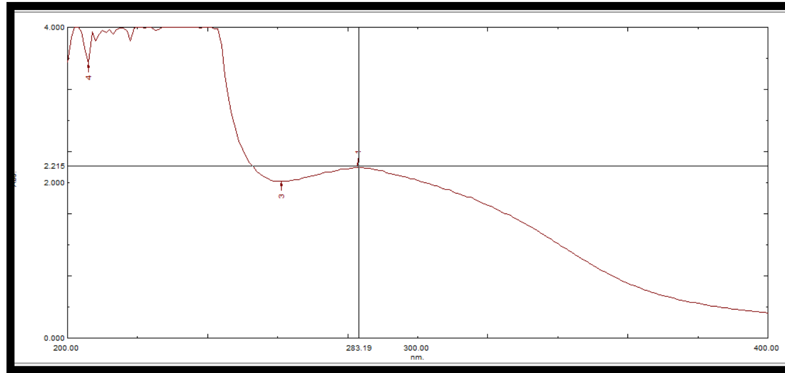
SAMPLE	MAX WAVELENGTH (nm)	OD
1	281.19	1.317
2	283.19	2.215
3	283.19	0.380
4	282.74	0.556

All the samples showed absorption maxima at 280nm which confirms the presence of PHA.

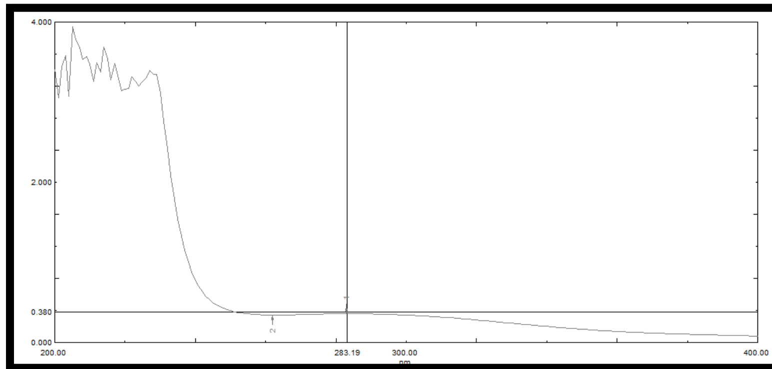
Graph 1:- SAMPLE 1 UV - Visible Spectroscopy



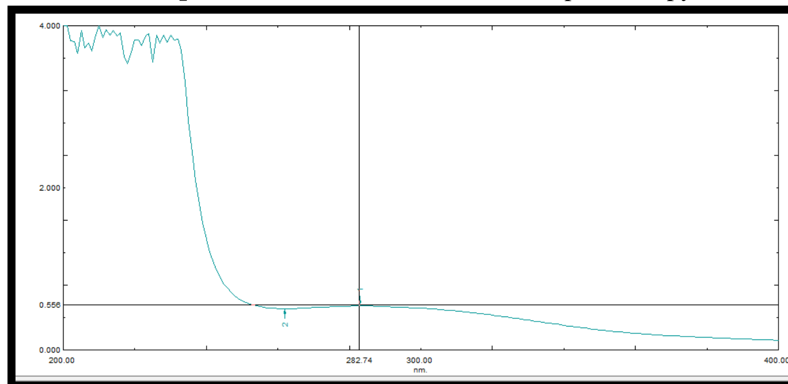
Graph 2:- Sample 2 UV – Visible Spectroscopy



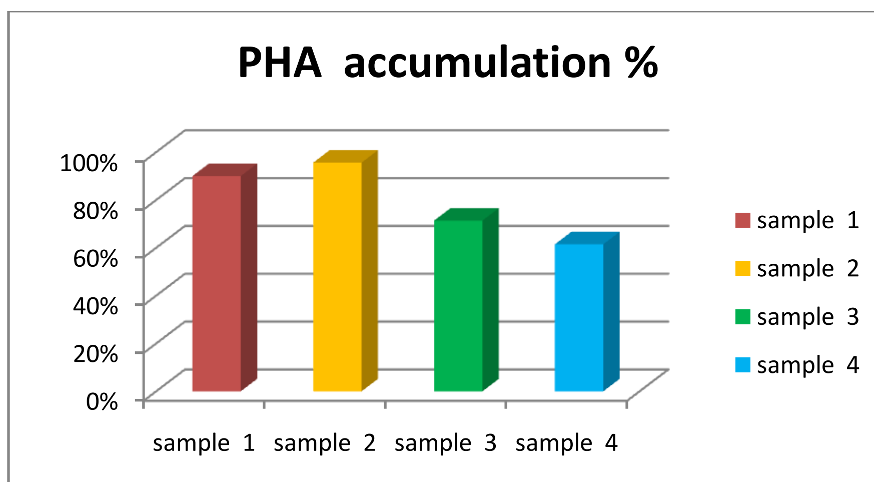
Graph 3:- SAMPLE 3 UV – Visible Spectroscopy



Graph 4:- SAMPLE 4 UV - Visible Spectroscopy



Graph 5:- PHA accumulation % in each sample



The PHA accumulation was found to be maximum in sample 2 followed by sample 1 , 3 and 4.

Discussion:-

PHA is a secondary metabolite produced by the various microbes present in nature. It can be of various types such as PHB, PHV, PHH and PHO [5]. The property of PHA being a biodegradable polymer makes it stand out of the crowd. But its high production cost compared to petrochemical - based plastics takes a back seat. Thus, during fermentation of microbes the biomass is produced in excess for the extraction of PHA. The aim is to find an easy and economic technique for the large scale production of PHA. Many common chemical methods using solvents have been used [8 & 23]. In certain cases, surfactants are used to recover the highly pure form of PHA. Out of the various novel techniques, alkali hydrolysis is a promising one as this proves to be comparatively simple and efficient. Various alkalis like sodium hydroxide, potassium hydroxide and ammonium hydroxide with varied ph can be used. It's important to note the effectiveness of the alkali in the digestion of non-PHA material without any distortion to the PHA. Retention of the properties of PHA depends on the technique of extraction. The efficiency of the production of PHA by microbes plays an important role in extraction. The microbes are isolated from various sites and are cultured in the minimal media. The minimal media induces the production of PHA in excess. The cells are then lysed and quantified for the PHA content. Sample 2 (sewage water) is the most promising source of PHA-producing bacteria (95.65%) compared to the other samples studied. Also highest accumulation is observed in waste containing samples. Therefore in future for large scale production, waste material can be utilized as PHA-producing bacteria's source.

Conclusion:-

Bioplastics can replace petroleum based plastics primarily due to its biodegradability. It is more convenient to use bioplastics as they do not lead to the pollution of the environment and can be used widely to decrease the level of pollution. The production source of bioplastic is better in waste containing samples which is significant for commercial production.

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