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Isolation and identification of new strains to enhance the production of biopolymers from marine sample in Karankura, Tamil Nadu

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ABSTRACT

Marine bacteria have many unexploited potential for novel products with a huge variability in structure and biological activity. Polyhydroxybutyrate is biodegradable thermo polyester which is synthesized by many bacteria under stress conditions they are reported to produce biopolymers like poly- β -hydroxybutyrate (PHB), which can be produced intracellularly as carbon and energy reserves. In this present study, five PHB producing bacterial colonies were isolated from the marine soil collected from the village Karankura, Thanjavur district. However only one bacterial colony is selected among others based on maximum PHB yield (0.701g/L). Bacteria from this colony was characterized by morphological, biochemical and optimization and identified as *Saccharococcus thermophilus*. The maximum PHB yield was recorded under the dry weight basis with different agro industrial wastes as the sole carbon source at pH 7 and temperature 50°C for 72h. Among the carbon sources and nitrogen used, molasses and ammonium sulphate yield more PHB yield respectively. However, acridine orange stained cells showed the presence of large quantities of granules in the cell cytoplasm when viewed under fluorescent microscope. The extracted polymer was compared with the standard PHB and was confirmed to be PHB using FTIR analysis.

Keywords: Poly- β -hydroxybutyrate, PHB, Molasses, FTIR

INTRODUCTION

Expansion of plastic production and consumption is having a significant impact both visibly and invisibly on the environment and society. Improper disposal of plastics has threatened natural environment worldwide since long time ago. Conventional petrochemical plastics are recalcitrant to microbial degradation [1]. Excessive molecular size might be mainly responsible for the resistance of these chemicals to biodegradation and their persistence in soil for a long time. These non-degradable petrochemical plastics accumulate in environment at a rate of 25 million tones per year [2]. To overcome this problem, the production and applications of eco-friendly products such as bio-plastics becomes inevitable.

Decades have been invested on extensive research to develop biodegradable polymers as a substitute for petrochemical based polymers due to their eco-friendly nature. Polyhydroxyalkanoates (PHA) are polyesters of

various R-hydroxyalkanoates and are considered as a good alternative amongst other biodegradable polymers developed, due to their biodegradability, biocompatibility, use of renewable resources as raw material, plastic and elastomeric material properties similar to petrochemical based polymers [3]. PHA's are accumulated as intracellular inclusion bodies by many Gram-positive and Gram-negative bacteria to levels as high as 90% of dry cell weight when carbon source is in excess but other nutrient supply (O, P, N and S) are in limiting condition [4]. As being biodegradable and biocompatible, a large number of PHA's and its copolymers have been exploited as bioplastics, biomedical applications such as drug delivery [5] tissue engineering [6] and in food packaging etc. Various researchers have isolated the promising micro-organisms from different environment such as municipal sewage sludge, marine microbial mats [7] and marine environments [8].

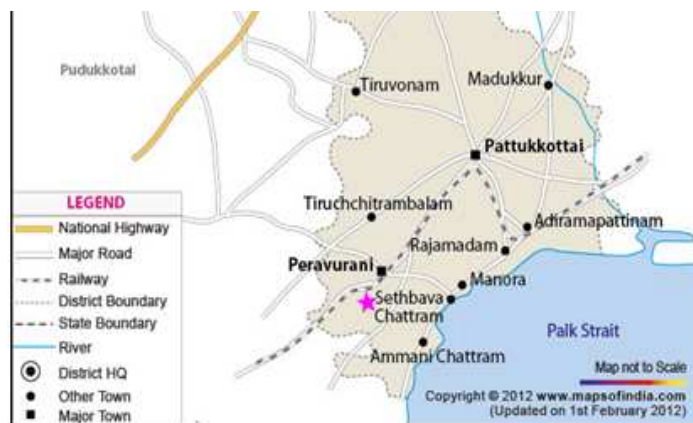
Typically, the bio-plastic production can be made by specific bacteria such as *Alcaligenes latus*, *Azotobacter vinelandii*, *methylotrophs*, *pseudomonas oleovorans* and etc. Polyhydroxyalkanoates (PHAs) which produced mainly by bacteria provide a degradable alternative to petrochemical plastics. So this study aimed to focus on production of Bio-plastics as they are the promising technology that can change the scenario of plastic waste management.

Ultimately, Sustainable product development has attracted a lot of attention in the last few years, and there has been extensive research looking at ways to provide material needs using energy efficient, non-toxic and renewable sources rather than finite materials. It is increasingly being realized that the use of long-lasting polymers for short-lived applications is not entirely justified, especially when increased concern exists about the preservation of finite resources.

Therefore, the strategies to upgrade and to optimize the PHA production using mostly available carbon sources have been chosen as research goal. Meanwhile, the production of PHA will be enhanced significantly. As marine environment is a prolific resource for the isolation of less exploited microorganisms, marine soil has been chosen as a sample. So the present study aimed to isolate PHB producing bacteria from marine environmental samples, screening of high PHB producers from the isolated bacteria, Optimization of cultural parameters for maximum PHB yields and Use of cheaper agro industrial substrates as a carbon sources for production of PHB.

MATERIALS AND METHODS

Collection of sample



* Sethbavachattaram Fig.1: Sample Collection Site.

Soil sample was collected from the village, Karankuru, Sethbavachattaram Taluk, Thanjavur District – Tamil Nadu. Soil sample was collected from the sampling station.

The collected sediment samples were first air dried at room temperature, then crushed using a porcelain mortar and pestle and then sieved for further analysis. The pH of the suspension was read using pH meter (Systronics, India), to find out the soil pH. Electrical conductivity of soil was determined in the filtrate of the water extract using Conductivity Bridge and Cation exchange capacity (CEC) of the soil was determined by using 1 N ammonium

acetate solution. The reagents used for the analysis were AR grade and double distilled water was used for preparation of solutions. The analyzed Physico-chemical parameters represented in Table.1 were observed by standard methods [9].

Isolation and identification of bacteria

The soil samples were passed through a sieve (1.7 mm mesh) to remove large pieces of debris and vegetation. The bacteria were originally isolated by plating dilutions of soils in saline solution (0.9% NaCl) on nutrient agar, was incubated at 37 °C for 48 h. The developed colonies were counted in plates and the average number of colonies per three plates was determined. The number of total bacteria (CFU) per gram dry weight soil was determined. Individual colonies of bacteria which varied in shape and color were picked up and purified by streaking on nutrient agar. The bacterial isolates were identified on the basis of classification schemes published in Bergey's Manual of Systematic Bacteriology [10] based on the characters such as morphology, physiology and nutritional, cultural characteristics and biochemical tests were presented in Table 2.

Rapid screening of native bacterial isolates for PHB production:

Sudan Black staining method:

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black B dye [11]. For rapid screening of PHB producers, nutrient agar medium supplemented with 1 % glucose was sterilized by autoclaving at 121°C for 20 minutes and cooled to 45°C. The medium was poured into sterile Petri plates and allowed for solidification. The plate was divided into 6 equal parts and in each part, a bacterial isolate was spotted. The plates were incubated at 30°C for 24 hours. Ethanolic solution of (0.02%) Sudan Black B was spread over the colonies and the plates were kept undisturbed for 30 minutes. They were washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue coloured colonies were taken as positive for PHB production. All the positive isolates were assigned the code numbers based on their source of isolation.

Quantification of PHB production and selection of isolate:

All the Sudan Black B positive isolates were subjected to quantification of PHB production [12]. A simplified media PYM media(g/L)containing only glucose, 20g, peptone,5g, yeast extract,3g and agar, 15g was used for PHB production. The bacterial cells containing the polymer were pelleted at 10,000 rpm for 10 min. and the pellet washed with acetone and ethanol to remove the unwanted materials. The pellet was resuspended in equal volume of 4% sodium hypochlorite and incubated at room temperature for 30 min. The whole mixture was again centrifuged and the supernatant discarded. The cell pellet containing PHB was again washed with acetone and ethanol. Finally, the polymer granules were dissolved in hot chloroform, allowed evaporating, and PHB weight was noted. Then concentrated 10 ml hot H₂SO₄ was added to the polymer granules. The addition of sulfuric acid converts the polymer into crotonic acid which is brown colored. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank. [13]. By referring to the standard curve, the quantity of PHB produced. was determined. Based on the PHB yields, 1 promising bacterial isolate were selected for further studies.

Residual biomass (g/L) = DCW (g/L) – Dry weight of extracted PHB (g/L).

$$\text{PHB accumulation (\%)} = \frac{\text{Dry weight of extracted PHB (g/L)} \times 100\%}{\text{DCW (g/L)}}$$

2.4. Fluorescence Staining Method (Acridine Orange)

Detection the isolate of PHB production was also done following fluorescent staining method using acridine orange [14]. 10 µl of 48 hr old culture of the isolate was transferred to an eppendorf tube containing 50 µl of acridine orange (Himedia) and incubated for 30 minutes at 30°C. After the incubation period, the culture was centrifuged at 4000 rpm, for 5 min. The pellet was collected and resuspended in distilled water. A smear was prepared on a clean microscopic slide and observed in a fluorescent microscope at 460 nm. The appearance of yellow coloured granules inside the cell indicates PHB production.

The bacteria positive for PHA production was selected by observing the granules under fluorescence microscope, Bacterial culture showing substantial fluorescence were selected for further study.

OPTIMIZATION OF pH AND TEMPERATURE FOR PHB PRODUCTION

Temperature Optimization

The culture was inoculated in the PYM medium and incubated at different temperature viz, 20° C , 30° C, 40° C and 50° C for temperature optimization. After 24h, 48h, 72h and 96h growth and cell dry weight was determined.

P^H Optimization

The culture was inoculated in the PYM medium and incubated at different p^H viz, 5, 6, 7, and 8 for PH optimization. After 24h, 48h, 72h, and 96h growth and cell dry weight was determined.

Source Optimization

Agro Industrial Sources:-

In Thanjavur district, molasses, coir pith, coconut flour, paddy chaff and vermicompost are available more and used as cattle feed or as fuel or manure in rural areas. As they contain more carbon content in different form, they were used in the present study as a crude carbon source after hydrolysis for PHB production.

TLC

About 50 ml of sample was loaded on the TLC plate and allowed to run in the solvent system consisting of ethyl acetate and benzene (SRL) (1:1) mixture for 40 min. for staining, 50 ml of iodine solution was vapourized in water both at 80-100°C. TLC plate was kept over the beaker containing iodine solution for 5-10 min in order for it to get saturated with iodine vapour. After 10 min black colour spots indicated the presence of PHB. The R_f value was measured.

Statistical Analysis

A 1-way analysis of variance (ANOVA) was carried out based on 3 replicates in order to establish statistical differences between different PHB yields for the different microorganism. (Table 4).

FTIR Analysis

In order to know the functional groups present in PHB extract, about 1mg extracted sample of PHB were dissolved in 5 ml of chloroform. Chloroform was allowed to evaporate to get PHB polymer film and was subjected to FTIR analysis by using FTIR spectrophotometer. Spectra were recorded in 4000 cm⁻¹ to 400 cm⁻¹ range.

TABLE.1 Soil Samples Analytical Report

S. No	Name of the Parameter	Sample Details
1	pH	7.56
2	EC (dsm ⁻¹)	0.52
3	Organic Carbon (%)	0.62
Available Macronutrients		
4	Available Nitrogen (Kg/ac)	95.6
5	Available Phosphorus (Kg/ac)	3.50
6	Available Potassium (Kg/ac)	135
Available Micronutrients		
7	Available Zinc (ppm)	0.85
8	Available Copper (ppm)	0.98
9	Available Iron (ppm)	4.85
10	Available Manganese (ppm)	3.26
Soil Fractions		
11	Clay	15.33
18.54Cat ion Exchange Capacity (C.Mole Proton⁺/Kg)		
Exchangeable Bases (C. Mole Proton⁺/Kg)		
12	Calcium	10.8
13	Magnesium	9.5
14	Sodium	2.16

Table 2 Biochemical Characteristics of the Bacterial Isolates

S. No.	Gram Staining	M	I	MR	VP	TSI	Citrate	Catalase	U	O	Glucose	Lactose	N R	Organism Name
1	+ve cocci	NM	-	+	-	Acid	+	+	+	+	+	+	-	<i>Saccharococcus thermophilus</i>
2	-ve cocci	NM	-	-	+	Acid gas	-	-	+	-	+	-	-	<i>Veillonella sp</i>
3	+ve rod	M	-	-	+	Acid, gas	-	+	+	+	+	-	-	<i>Curtobacterium sp</i>
4	+ve cocci	NM	-	-	+	Acid, gas	-	-	+	+	+	+	-	<i>Sarcina sp</i>
5	-ve cocci	M	-	+	-	Acid, gas	-	-	+	-	+	-	-	<i>Syntrophococcus sp</i>

M- Motility I-Indole MR-Methyl Red U- Urease O- Oxidase NR- Nitrate Reduction

Table 3 PHB yield by the selected Bacterial isolates

S. No	Name of the Organism	CDW(g/l)		PHB(g/l)		%PHB	
		Mean	Std.dev	Mean	Std.dev	Mean	Std.dev
1	<i>Saccharococcus thermophilus</i>	0.993	± 0.124	0.601	± 0.028	64.80	± 10.416
2	<i>Veillonella sp</i>	1.475	± 0.035	0.052	± 0.009	3.50	± 0.565
3	<i>Curtobacterium sp</i>	3.399	± 0.117	0.431	± 0.057	12.699	± 2.121
4	<i>Sarcina sp</i>	1.121	± 0.239	0.346	± 0.008	31.515	± 5.989
5	<i>Syntrophococcus sp</i>	1.100	± 0.043	0.531	± 0.029	48.185	± 0.741

Table 4 ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.363374	4	0.090843	1227.614	1.15E-07	5.192168
Within Groups	0.00037	5	0.000074			
Total	0.363744	9				

F0.05 df 4 and 5 = 6.26

Since calculated value 1227 is greater than the table value (6.26), there is a significant.

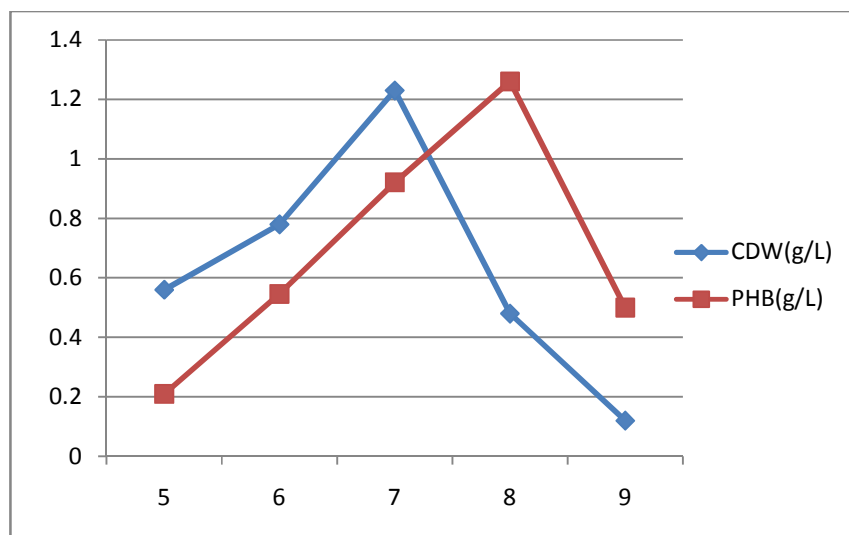
Fig3. Effect of p^H on PHB production

Fig 4. Effect of Temperature on PHB production

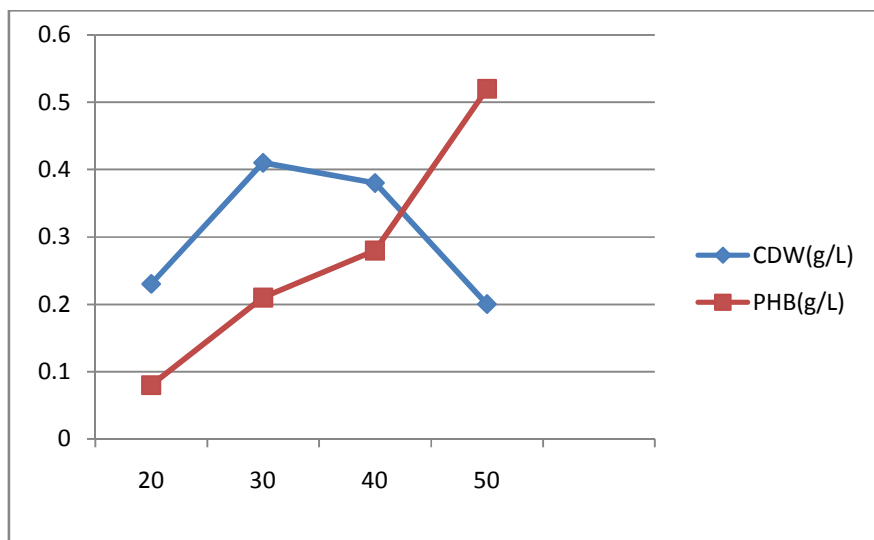


Fig 5. Effect of Incubation time on PHB production

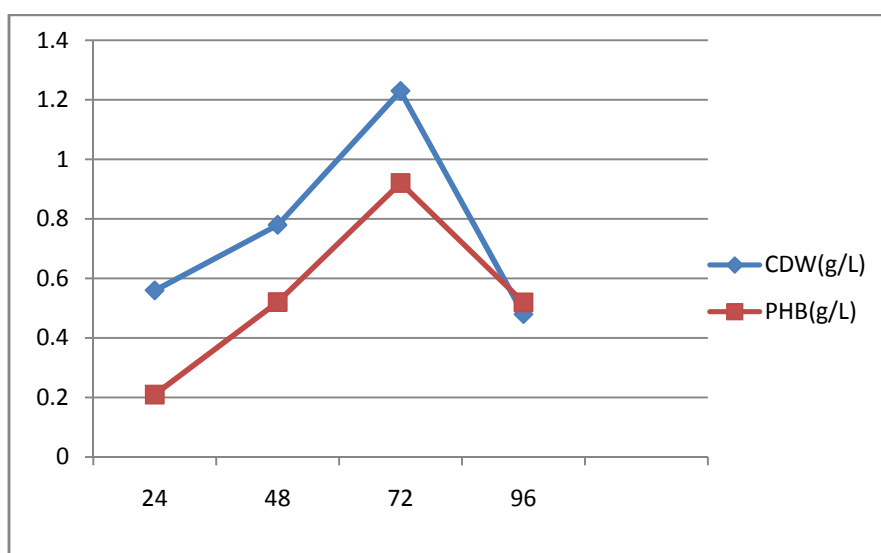


Table 6. Effect of Carbon sources on PHB production

S. No.	Carbon source	CDW(g/L)	PHB(g/L)	%PHB
1	Molasses	0.805	0.581	72.17
2	Coir pith	0.612	0.371	60.62
3	Coconut flour	0.557	0.321	57.63
4	Vermicompost	1.204	0.482	40.0
5	Paddy chaff	0.756	0.442	58.46

Table 7. Effect of Nitrogen sources on PHB production

S. No.	Nitrogen source	CDW(g/L)	PHB(g/L)	%PHB
1	Beef Extract	0.521	0.308	59.11
2	Casein	1.212	0.432	35.64
3	Ammonium sulphate	0.993	0.601	60.52
4	Ammonium nitrate	0.630	0.253	40.15
5	Malt Extract	0.916	0.448	48.90

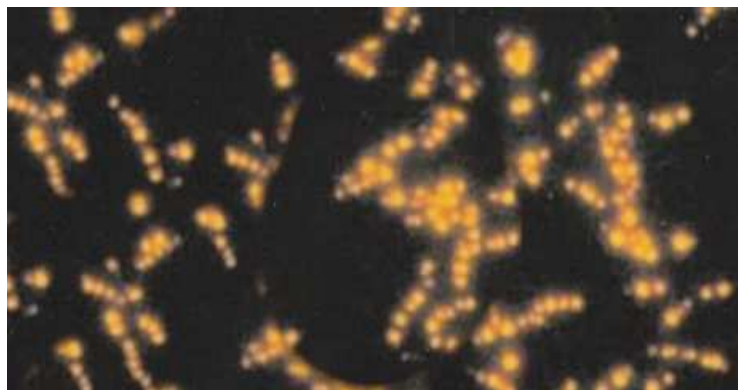
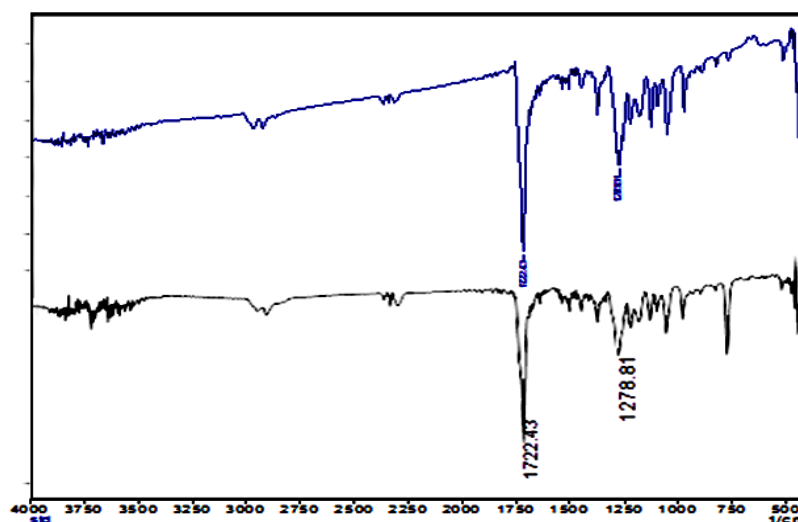
Fig 2. Acridine orange staining of *Saccharococcus thermophilus*

Figure 3 . FTIR spectra of PHB Standard (blue) and extracted PHB (black).



RESULTS AND DISCUSSION

The secret of marine bacterial isolates have more expectations in search of new bioactive compounds as mentioned earlier. So in this present study, the soil sample was collected from the village Karakura near Sethubavachatram, Thanjavur district (Fig 1). The soil physico-chemical parameters analysed were given in table 1 used to investigate how these parameters influence the bacterial diversity. Because, microbial survival in soils depends on intrinsic biochemical and structural properties, physiological, and/or genetic adaptation including morphological changes of cells, as well as environmental modifications of metal speciation as described by [15].

Based on microscopic and biochemical observations, isolates were identified as *Saccharococcus thermophilus*, *Veillonella sp*, *Curtobacterium sp*, *Sarcina sp*, *Syntrophococcus sp* (Table 2).

The isolates were tested for PHB production following the viable colony screening method based on the intensity of staining. All the 5 isolates gave the positive result for PHB accumulation through Sudan Black B staining method scoring. All the 5 Sudan Black B positive isolates were subjected to quantitative estimation of PHB production. The synthesis of PHB was noticed from the log phase of growth and it continued until late exponential phase as the carbon source was utilized for both growth and PHB production. The substantial PHB production using the simplified glucose peptone medium may be attributed to the presence of complex organic nitrogen source, peptone

favouring the growth as well as PHB accumulation. They produced PHB yields of 0.601 0.052 0.431 0.346 0.531 g/100 ml, respectively as given in Table 3.

A 1-way analysis of variance (ANOVA) was carried out based on 3 replicates in order to establish statistical differences between different PHB yields for the different microorganism. (Table 4). $F_{0.05, df 4 \text{ and } 5} = 6.26$ Since calculated value 1227 is greater than the table value (6.26), there is a significant was defined at $P < 0.05$. Based on the highest yield, *Saccharococcus thermophilus*, was selected for further studies.

The effects of culture conditions on PHA production and showed that growth conditions including pH, temperature and carbon source plays an important role in the production rate of PHA [16]. The effect of pH of the medium on PHB production was assessed. The data revealed that pH 8.0 was found to be optimum to get more PHB. The pH 8.0 has recorded the mean PHB of 1.32g /100 ml (Fig 3). This was in agreement with [16] who observed that the PHB in *Saccharococcus thermophilus* strain grown on luminescence marine media adjusted to pH 8.0 the yield of PHB in these cells was 1.62 g /100 ml in 12.074 of cell dry weight. The effect of temperature and incubation time on PHB production was evaluated. The data revealed that temperature 50⁰c and 76 hour were found to be optimum to get more PHB respectively (Fig.4&5). Isolation of new strains capable of utilizing the cheap carbon source is essential to reduce the cost is the major concern at industrial level.[17]

To study the effects of carbon and nitrogen sources and to select the best source for maximum PHB production, different agro industrial carbon and nitrogen sources were used. (Table 6&7). The agro industrial carbon sources were chosen based on availability and accessibility in and around thanjavur district. Among the carbon sources used (Table 4) molasses yielded more PHB 0.581g/L in 0.805 g/L of CDW as supported by [18]. Because of more cellulose content other carbon sources not yield much more PHB when compared with molassess. Among the nitrogen sources used, ammonium sulphate was observed as the best supporter of PHB production (0.601g /100 ml). These results are in agreement with the results obtained by [19] who also observed the highest PHB production (2.260g 100 ml⁻¹) by *R. eutropha* on MSM medium supplemented with ammonium sulphate. [20] [21] also worked on the accumulation of PHB by *A. eutrophus* with ammonium sulphate. Black colour bands were observed in TLC and Rf value was measured and calculated 1.10 which indicated the presence of PHB in the production medium.

To investigate about the functional groups present in PHB and for the conformation FTIR Spectroscopy study was carried out with standard PHB (sigma).FTIR spectra of the extracted polymer show peaks at 1726.29cm⁻¹ and 1276.88cm⁻¹ corresponding to specific rotations around carbon atoms specific to certain functional groups (Fig. 3). The peak at 1726.29 cm⁻¹ corresponds to C=O stretch of the ester group present in the molecular chain of highly ordered crystalline structure [22]. The peak at 1276.88cm⁻¹ corresponds to -CH group. These peaks are corresponding to the peaks obtained for the standard PHB (Sigma) at 1728cm⁻¹ and 1282cm⁻¹ exactly confirming that the extracted polymer is PHB.

CONCLUSION

The organic synthesis is one of the major role of research in science, from plastics to medication it participates in the improvements of everyone life [23]. So, biopolymers produced by different microorganisms are getting very much importance both in agriculture, economics and health sciences. Among the biopolymers that are industrially important are bioplastics. As they can be produced from renewable resources, which are recyclable [24] there is a need to optimize the conditions for high production of biopolymers. In this present study, the soil of Karankura, near sethbachatram located in east coast of Thanjavur District have been investigated to isolate and identify new strains in order to enhance the production of PHB.

The production of PHB from the isolate depending on different factors such as the following:

The existence of carbon source where the sugar of molasses was considered the best carbon source to produce PHB compared to the other carbon sources. The presence of nitrogen source, ammonium sulphate was investigated as the best source of nitrogen that help to produce PHB compared the others. Soil pH of the neutral range which is closed to pH 8.0 was considered to be the best value that helps the isolates to produce the PHB compared to the other pH of 7 and 9. Further studies needs the molecular identification and scale up studies.

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