Original Research Article

Enhancement of Technique for Optimized Production of PHA from Marine Bacteria, Utilizing Cheaply Available Carbon Sources at Thanjavur District, India

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A B S T R A C T

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Global environment concerns and solid waste management complications have generated much curiosity in the development of biodegradable plastics. Polyhydroxyalkoanates are biodegradable polyesters are synthesized by many bacteria. In pursuit of new bioactive entities, investigations are being expanded to marine habitats. In the present study, the soil sample was collected from unexploited coastal area Sethubavachatram, Thanjavur District at four different seasons. Determination of 25 diverse bacterial species showed the predominance of 13 genera. Seasonal variations of soil physico-chemical parameters were investigated. Then the efficacy of PHA accumulating bacteria was screened by Sudan blank staining. Followed by quantification of PHA, three isolates such as KSN1, KSN2 and KSN5 accumulated more. In our proposed study, KSN1 and KSN2 were evidenced for the first time can accumulate more PHA. To enhance the PHA yield, UV mutagenesis was performed. Among the agro industrial wastes used, molasses was chosen as the best carbon source as KSN5 *Bacillus* sp. yielded 95% of PHA with 20.54±0.14 g/L CDW. Likewise, ammonium sulphate served as the best nitrogen source for 73.81% (CDW12.18±0.01 g/L) by KSN5 *Bacillus* sp. The PHA obtained from these isolates was validated and16S rRNA gene sequencing was performed.

Introduction

Pollution is a condition in which contaminants are introduced in to the natural environments leading to adverse changes in the surroundings and human activity is the main cause for the same. It may arise in different geographical locations leading to alterations in soil, water or air. Pollutions may lead to critical problems in the global geochemical cycles as well as the sustainable habitation of humans as well as 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.

Plastics are the foremost noxious waste, but they are considered to be a bubbly métier for humanity. Today accumulation of non-degradable plastic bags in the environment is one of the main causes for pollution. Flechter (1993) stated that conventional petrochemical plastics are recalcitrant to microbial degradation. 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 tons per year (Lee, 1996). The Central Pollution Control Board (CPCB) reported that India generates 56 lakh tonnes of plastic waste annually, with Delhi accounting for a staggering 689.5 tonnes a day and "Total plastic waste which is collected and recycled in the country is estimated to be 9,205 tonnes per day (approximately 60% of total plastic waste) and 6,137 tonnes remain uncollected and littered". Most plastics are made out of petroleum products and so plastics production has an impact on petroleum consumption.

The total oil consumption of the world in 2008 was 87.2 million barrels a day. Known oil reserves total 1.24 trillion barrels, which at the current rate of consumption would last 41 years. The plastics industry had the third highest carbon emissions in the chemical sector behind industrial organic chemicals and industrial inorganic chemicals. Incineration of plastics is potentially dangerous and can be expensive. United Nations Environment Programme estimates that there are 46,000 pieces of plastic litter floating in every square mile of ocean. Animals often mistakenly ingested the plastics as feed. The ingested plastics are clogging their intestine which results in death by starvation. Dioxin, a highly carcinogenic and toxic by-product of the manufacturing process of plastics, is one of the chemicals believed to be passed on through breast milk of the mother to the nursing infant.

An alternate to plastics – Bioplastics

Bio-plastics are bio-based, biodegradable plastics with almost similar properties to synthetic plastics. Biodegradation can be explained as a chemical process during which micro-organisms that present in the environment convert materials into natural substances such as water, carbon dioxide, and compost.

We have identified five major advantages of bioplastics in this note as follows,

- Potentially a much lower carbon footprint
- Lower energy costs in manufacture
- Do not use scarce crude oil
- Reduction in litter and improved compostability from using biodegradable bioplastics
- Improved acceptability to many households.

The success in the biodegradable plastic strategy largely depends on the isolation of potent PHA accumulating microbes and optimizing culture parameters for its maximum biosynthesis. Keeping these realities as objective, the proposed work was intended to achieve.

Materials and Methods

Description of sampling station

About four soil samples were collected from the coastal area, Sethubavachatram, Thanjavur District – Tamil Nadu (Fig.1). Thanjavur District is one of the 32 districts of the state of Tamil Nadu, in southeastern India. It is located between latitude

9°38'58"N and longitude 78°57'38"E. This district has about 45km length of coastline in the southeastern part.

Sampling schedule

Soil sample was collected from the sampling station seasonally for a period of one year from Jan-2010 to Dec-2010. The climate is monsoonic and the calendar year has been divided into four season viz., Post monsoon (January - March), summer (April - June), Pre monsoon (July - September) and Monsoon (October- December).

Isolation of marine soil bacteria

1gm of soil samples were taken and mixed in 10 ml distilled water in test tubes. Serial dilution technique was performed.0.1ml aliquot of various dilutions (10^{-5}–10^{-6}) was spread on a nutrient agar medium (peptone-5g,sodium chloride-5g, yeast extract -1.5g ,beef extract-1.5g, agar-15g/L pH-7.2) and incubated at 37^0C for 2 days.

Identification and characterization of marine bacterial isolates

Morphological analyses of the colony of bacterial isolates were studied based on the color, shape, size and margin of the colonies. Morphological tests were done by the standard procedures given by Barthalomew and Mittewer (1950).

Physico-chemical analysis of soil sample

The soil samples were collected in zip-lock polythene bags from selected study site for the period of one year from Jan-2010 to Dec-2010. 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 as described by Jackson (1973). Organic carbon content was determined by Walkley and Black (1934). Available nitrogen was estimated by alkaline permanganate method as described by Subbiah and Asija (1956) and available phosphorus by Brayl method as described by Bray and Kurtz (1945). The available calcium in the extract was determined by Versenate method (Jackson, 1973). Other nutrient based parameters i.e. available phosphate and total nitrogen were estimated using standard methods of APHA (1987). Other nutrients such as magnesium, sodium and available iron were analyzed following the method of Muthuvel and Udayasoorian (1999).

Screening methods for PHA accumulation in Bacterial isolates

(a) Preparation of inoculum

The primary inoculum was prepared in nutrient medium in 250 ml conical flask containing 50 ml of sterile medium and inoculated from the stock culture. The fresh overnight culture was used as an inoculum for production of PHA.

Then the nutrient medium was prepared and sterilized in autoclave at 121°C for 15 minutes.100 ml of production medium was prepared in 250ml conical flask and sterilized. 1 % inoculum was transferred aseptically into the production medium and incubated at 37°C for 48 hours. The medium was agitated at 100 rpm for better aeration and growth of the organism.

(b) Rapid screening of PHA accumulating bacterial isolates

Sudan black staining method

All the bacterial isolates were qualitatively

tested for PHA production following the viable colony method of screening using Sudan Black B dye (Juan *et al.,* 1998). Detection the isolate of PHA production was also done following fluorescent staining method using acridine orange, as suggested by Senthilkumar and Prabhakaran (2006).

Mass production of PHA by constructive bacterial isolates

One liter of peptone yeast extract medium(Glucose - 20g, Peptone - 5g ,Yeast extract - 3g, Agar - 15g, Distilled water-1000ml) 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 screened positive isolates of PHB producers were inoculated and incubated for 24 hours at room temperature for each 50 ml of medium.

Extraction and quantitative assay

The spectrometric chemical assay for the determination of PHB from the sample was estimated by using Slepecky and Law (1961) method**.**

PHA accumulation (%) =

$$\frac{\text{Dry weight of extracted PHA (g/L)} \times 100\%}{\text{CDW (g/L)}}$$

Statistical analysis

All measurements were performed in triplicate, and all values reported are the mean of three replicates. The PHA accumulated by the screened isolates was processed using Analysis of variance (ANOVA).The significance level was chosen to be 0.05 (or equivalently, 5%) by keeping in view the consequences of an error.

Selection of potential PHA accumulating bacterial isolates

The PHA production ability of positives isolates were studied in the PY standard production medium was studied. Among the isolates, 3 which accumulated the maximum PHA were chosen for further optimization studies.

Optimization of PHA production

pH optimization (Nakata, 1963)

The selected 3 bacterial isolates were inoculated in the PY medium and incubated at different pH viz., 4,5,6,7 and 8 separately for pH optimization. After 24h, 48h, 72h and 96h growth and cell dry weight and PHA were determined.

Temperature optimization (Grothe *et al.,* 1999)

The selected 3 bacterial isolates were inoculated in the PYM medium and incubated at different temperature viz., 20° C, 30° C, 40° C and 50° C separately for temperature optimization. After 24h, 48h, 72h and 96h growth and cell dry weight and PHA were determined.

Incubation time optimization (Nakata, 1963)

The selected 3 bacterial isolates were inoculated in the PYM medium and incubated at different time intervals viz., 24h, 48h, 72h and 96h separately. After each incubation period cell dry weight and PHA were determined.

Source optimization

Agro industrial sources

In Thanjavur district, molasses, coir pith,

coconut oil cake, 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 PHA production. The afore said wastes were collected from Arignaranna sugar factory, Kurungulam, Coir factory, at Vallam, local oil mill, Paddy processing unit and Sriamman Biocare, Thanjavur respectively.

Validation of the PHA

Thin Layer Chromatography

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 vaporized 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, dark green colour spots indicated the presence of PHA.

Gas chromatography mass spectroscopy

GC analysis of powered PHA was carried out by the method described by Brandl *et al.* (1988) was conducted at IICPT, Thanjavur. About 2mg sample was subjected to methanolysis with a solution consisting of 1ml chloroform, 0.85ml methanol and 0.15ml H_2SO_4 at 100°C for 140 min. Deionized water was added to the cooled samples, the contents were homogenized and the bottom phase was used for GC analysis. The methyl esters were analyzed by GC flame ionization detector in a 30 m DB-1 capillary column was used. N_2 was used as carrier gas. The injector and detector are at 170°C and 220°C respectively. The

program was used for 55°C for 7 min; ramp of 4°C per min up to 100°C; 10°C per min rise up to 200°C and hold at 200°C for 10 min.

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 sethubavachatram, Thanjavur district. The soil physico-chemical parameters analysed were given in Table 1 used to investigate how these parameters influence the bacterial diversity.

Wuertz and Mergeay (1997) described that microbial survival in soils depends on intrinsic biochemical and structural properties, physiological, and genetic adaptation including morphological changes of cells, as well as environmental modifications of metal speciation.Based on microscopic and biochemical observations, 13 diverse bacterial isolates were identified.

The isolates were tested for PHA production following the viable colony screening method (Juan *et al.,* 1998) based on the intensity of staining. The 5 isolates such asKSN1 *Saccharococcus thermophiles* sp., KSN2 *Syntrophococcus* sp., KSN3 *Veillonella* sp., *Curtobacterium* sp., KSN4 *Sarcina* sp., KSN5 *Bacillus* sp. gave the positive result for PHA accumulation through Sudan blank staining method scoring.

All the 5 Sudan Black B positive isolates were subjected to quantitative estimation of PHA production. The synthesis of PHA 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 PHA production. The substantial PHA

production using the simplified glucose peptone medium may be attributed to the presence of complex organic nitrogen source, peptone favoring the growth as well as PHA accumulation (Page and Knosp, 1992; Song and Yoon, 1999; Thakur, 2002). They produced PHA yields of 0.901±0.01g/L, 0.642±0.05, 0.422±0.01, 0.941±0.10and 15.631±0.01g/L, respectively.

A 1-way analysis of variance (ANOVA) was carried out based on 3 replicates in order to establish statistical differences between different PHA yields for the different microorganism. F0.05 df 4 and 5 = 6.26 Since calculated value 1227 is greater than the table value (6.26), there is a significance was defined at $P < 0.05$. Based on the highest yield, KSN5 *Bacillus* sp. was selected for further studies.

Pozo *et al.* (2002) studied 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. The effect of pH of the medium on PHA production was assessed. The data revealed that pH 8.0 was found to be optimum to get more PHA. The pH 8.0 has recorded the mean PHA of 1.33g /100 ml (Fig. 2).

This was in agreement with Sharmila *et al.* (2011) who observed that the PHA in *Saccharococcus thermophilus* strain grown on luminescence marine media adjusted to pH 8.0 the yield of PHA in these cells was 1.62 g /100 ml in 12.074 of cell dry weight. The effect of temperature and incubation time on PHA production was evaluated. The data revealed that temperature 50^0C and 76 hour were found to be optimum to get more PHA respectively (Fig. 3&4). Steinbuchel (1995) stated that isolation of new strains capable of utilizing the cheap carbon source

is essential to reduce the cost is the major concern at industrial level.

The agro industrial carbon sources were chosen based on availability and accessibility in and around Thanjavur district. Among the carbon sources used (Table 3) molasses yielded more PHA10.810g/L in 0.905 g/L of CDW as supported by Senthilkumar and Prabhakaran (2000).

Among the nitrogen sources used, ammonium sulphate was observed as the best supporter of PHA production (10.801g/L) presented in Table 4. These results are in agreement with the results obtained by Khanna and Srivastav (2005) who also observed the highest PHA production (2.260g 100 ml^{-1}) by *R. eutropha* on MSM medium supplemented with ammonium sulphate. Mulchandani *et al.* (1989) and Raje and Srivastav (1998) 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 1.09 which indicated the presence of PHA in the production medium.

By investigating the potential fragmentation patterns and the molecular mass of the fragments, the identities of specific peaks in GCMS spectra were correlated to that of carbonyl and hydroxyl ends of the corresponding alkanoates. In the chromatograms of KSN5 *Bacillus* sp. 9 peaks are obtained in which n-Hexadecanoic acid, methyl esters was noted at the retention time of 16.71min n-Hexadecanoic acid at 17.32 min and 1,2-Benzenedicarboxylic acid, diisooctyl ester at 26.30 min retention time. These compounds signify that the monomer chains were of biodegradable polyester family. The n- hexadecanoic acid is an aliphatic polymer esters.

Table.1 Physico-chemical parameters of soil sample

S.No	Name of the Parameter	Sample Details
1	pH	7.26
2	EC (dsm^{-1})	0.53
3	Organic Carbon (%)	0.61
Available Macronutrients		
4	Available Nitrogen (Kg/ac)	95.5
5	Available Phosphorus (Kg/ac)	3.40
6	Available Potassium (Kg/ac)	132
Available Micronutrients		
7	Available Zinc (ppm)	0.82
8	Available Copper (ppm)	0.96
9	Available Iron (ppm)	4.81
10	Available Manganese (ppm)	3.22
Soil Fractions		
11	Clay	15.30
Cat ion Exchange Capacity (C.Mole Proton^{+}/Kg) = 18.54 Exchangeable Bases (C. Mole Proton^{+}/Kg)		
12	Calcium	10.5
13	Magnesium	9.1
14	Sodium	2.12

Table.3 Effect of carbon sources on PHA production

S.No.	Carbon source	PHA(g/L)	%PHA
1	Molasses	10.81±0.01	72.17
2	Coir pith	0.381±0.05	60.65
3	Coconut flour	0.325±0.10	57.64
4	Vermicompost	0.472±0.01	40.05
5	Paddy chaff	0.432±0.00	57.46

Table.4 Effect of Nitrogen sources on PHA production

S.No.	Nitrogen source	PHA(g/L)	%PHA
1	Beef Extract	0.907±0.01	59.10
2	Casein	0.711±0.03	35.51
3	Ammonium sulphate	10.801±0.00	60.62
4	Ammonium nitrate	0.943±0.10	40.11
5	Malt Extract	0.440±0.01	48.80

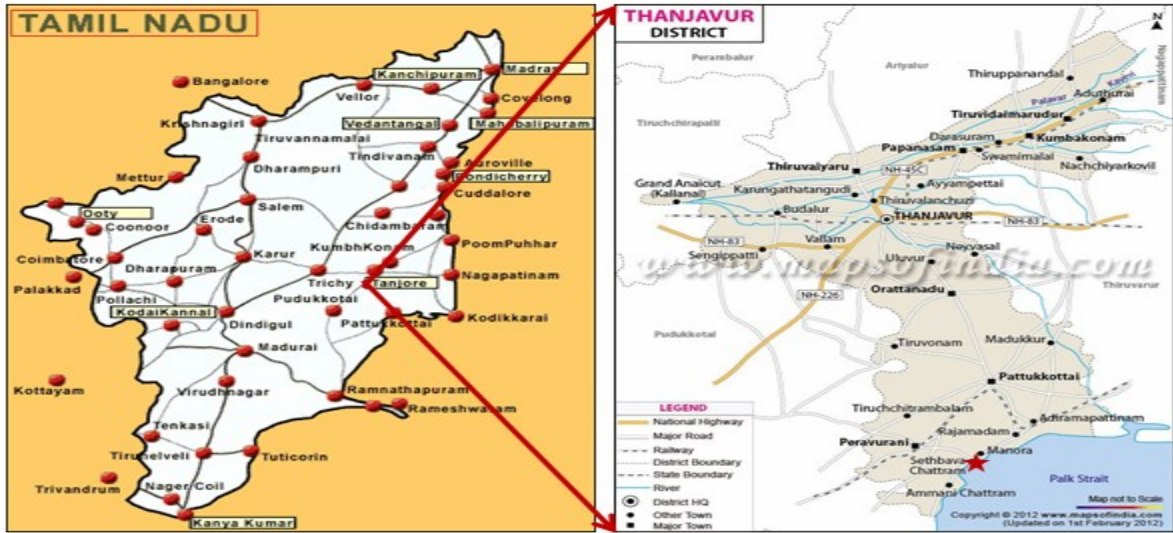**Fig.1** Sample Collection Site   Sethubavachatram

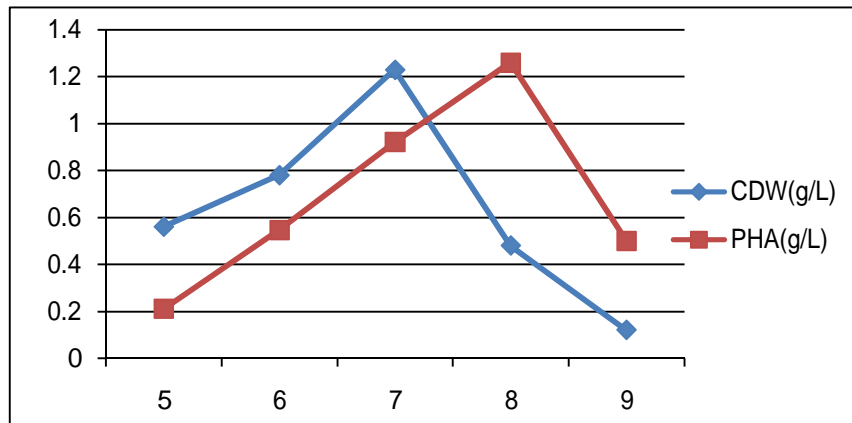**Fig.2** Effect of pH on PHA production
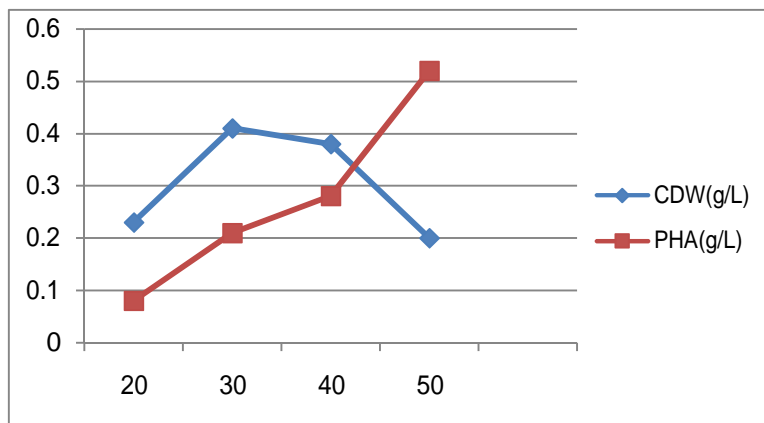

Fig.3 Effect of Temperature on PHA production

Fig.4 Effect of Incubation time on PHA production
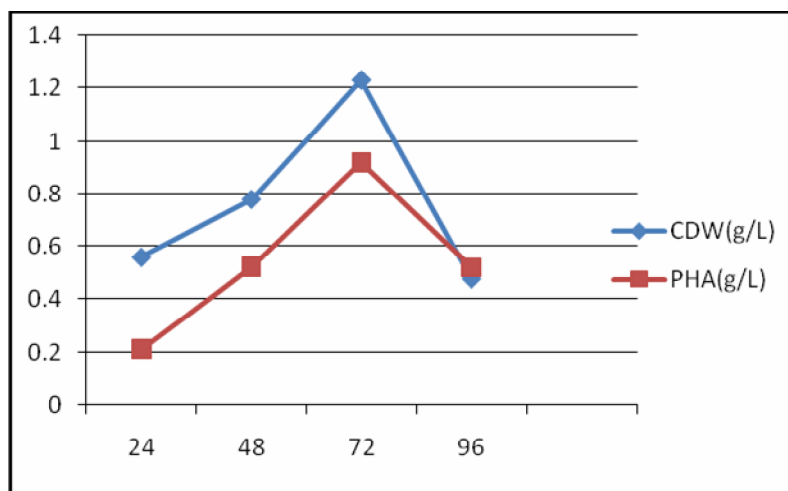
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