

Isolation and Characterization of PHA Producing Bacteria from Sewage Samples of Assam

Jasmine Chetia



Abstract: Polyhydroxyalkanoate (Pha) is a family of naturally occurring polymers which is reportedly produced by more than 75 genera of Gram-positive and Gram-negative bacteria. Although the industrial production of this bioplastic commenced decades ago, its large scale production is still debilitated by factors such as rate of production by the bacteria and the raw materials for its production, which finally elevates the production cost. The present thrust in this area is to isolate bacterial species which have a high turnover of pha with minor expenditure. Sewage and sewage sludge samples are rich sources of microflora which offer a nutrient limited habitat to the individual organisms. Under these conditions of stress, native organisms develop a metabolomics which efficiently convert vital nutrients into essential macromolecules, and in this case a storage polymer of industrial significance i.e. pha. In this pilot study an attempt was made to isolate indigenous bacterial with ability to produce the polymer.

Keywords: Pha, Indigenous Bacteria, bio plastic, sewage

I. INTRODUCTION

Poly- β -alkanoates (PHA) has proving itself as an advance polymeric material as well as an alternative for plastic material. The only hinge stopping large scale utilization of PHA at industrial scale and in daily lives is its, economical production. Large scale economical production of polyhydroxybutyrate (PHB) depends upon optimization and selection of high PHA producing strain (Madhuri Girdhar, 2014). PHA is accumulated naturally in bacteria to store carbon and energy in nitrogen and phosphorus deficient condition (Verlinden et al., 2007). PHAs are separated into three classes: short chain length PHA (scl PHA, carbon numbers of monomers ranging from C3 to C5), medium chain length PHA (mcl PHA, C6–C14), and long chain length PHA (lcl PHA, >C14) (Zinn et al., 2001).

There have been several attempts to isolate high yielding bacterial PHA producers. Liu *et al.*; isolated two novel strains of gram positive bacteria capable of accumulating poly(3-hydroxybutyrate co 3-hydroxyvalerate) from an anaerobic-activated sludge system, fed with acetate which also accumulated acetate or glucose under anaerobic conditions. 16sRNA sequencing revealed that both strains belonged to the gram-positive high-G+C group and accumulated 14.4% (cell dry weight) polyhydroxyalkanoate (Liu et al., 2000).

The microbial community composition of polyhydroxyalkanoate producing organisms (PHAAOs) was investigated by Oshiki *et al.*; in an aerobic waste water treatment plant using FISH (Fluorescence in situ hybridization) and Nile blue A staining techniques.

The PHB production by the bacillus strains isolated from soil was found to be growth associated in a study conducted by Aarhi *et al.*; at Mysore. Based on their 16S rRNA gene sequences, the bacteria were identified as *Bacillus mycoides* DFC1, *Bacillus cereus* DC1, *Bacillus cereus* DC2, *Bacillus cereus* DC3 and *Bacillus cereus* DC4. The polymer production by the strains was found to vary from 12.18% to 57.2 % content (w/w) of the dry cell weight (Aarhi, 2011). With an aim to isolate PHA producers, Shamala *et al.*; screened 123 isolates of which 12 isolates were found to be positive. All the isolates were identified to be various strains of Bacillus which producing 11-41% of PHA in sucrose containing medium over a growth period of 24-74 hours. An analysis of the polymer using NMR spectroscopy, infrared spectroscopy and gas chromatography indicated that the polymer contained polyhydroxybutyrate and polyhydroxyvalerate (Shamala et al., 2003).

In 2008, Vishnuvardhan *et al.*; screened sewage samples and sludge as a source to isolate bacteria which produce PHA granules. Of the 105 isolates out of which 15 were positive for PHA production with 13 isolates to produce PHB and 2 isolates were producing polyhydroxybutyrate – co-polyhydroxyvalerate (Vishnuvardhan Reddy et al., 2008).

In a bid to understand the role of nutrient limitation and produce PHA using cheap raw material Shamala *et al.*; studied the co-production of polyhydroxyalkanoates (PHA) and α -amylase by *Bacillus* sp CFR67 using corn starch and other agro-industrial waste as a substrate. Bacterial growth and polymer production was elevated by supply of hydrolysates of wheat bran and rice bran. In batch culture there was production of Polyhydroxy butyrate co-polyhydroxyvalerate in the ratio 95:5 (Shamala et al., 2012). In a similar approach, Bhattacharya *at el* reported the production of poly-3-(hydroxybutyrate-co-hydroxyvalerate) by *Haloferax mediterranei* using rice-based ethanol stillage which holds promise for competitive production of PHB using cheap carbon sources in industrial scale (Bhattacharyya et al., 2014). Reddy *et al.*; investigated the influence of substrate load and nutrient concentration (nitrogen and phosphorous) on PHA production using wastewater as substrate and mixed culture as biocatalyst. PHA accumulation was high at higher substrate load [OLR3, 40.3% of dry cell weight (DCW)], low nitrogen (N1, 45.1% DCW) and low phosphorous (P1, 54.2% DCW) conditions. With optimized nutrient conditions production efficiency increased by 14%. (Venkateswar Reddy and Venkata Mohan, 2012).

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* Correspondence Author

Jasmine Chetia*, Department of Botany, Sonari College, Sonari, Assam, India

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Nutrient stress is an important factor which triggers the accumulation of PHA. For instance in their study Phanse *et al.*; observed that there was more PHB producing bacteria in sewage and activated sludge than soil samples. Out of 23 bacterial strains isolated there was a ratio of 14:9 with respect to sewage: soil. (Phanse *et al.*, 2011).

Waste from Pulp, Paper, and Cardboard Industry have been also explored as a potential raw material for the production of PHA. Luhana *et al.*; reported isolation of PHA producing bacteria which accumulate upto 80% of PHA of their total dry cell weight after 48 hours inoculation from paint industry effluents (Luhana K. K., 2013). The effect of chemical oxygen demand (COD)/N ratio on PHB accumulating ability in an anaerobic/aerobic cycle sequential batch reactor was studied by Liu *et al.* The population of the activated sludge was studied periodically using denaturing gradient gel electrophoresis (DGGE). The predominant strains belonged to five genera: *Bacteroidetes sp.*, *Acinetobacter sp.*, *Betaproteobacteria sp.*, *Gammaproteobacteria sp.* (Liu *et al.*, 2013).

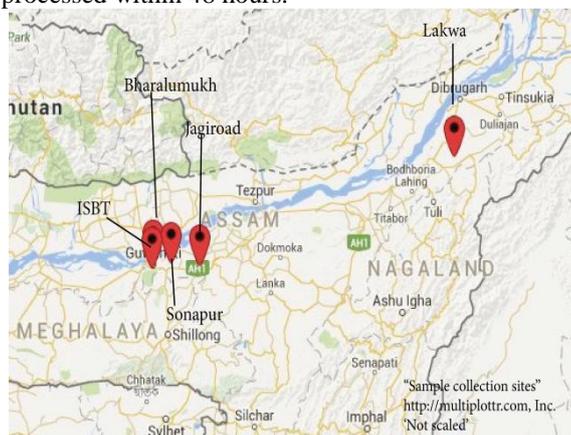
In 2002 Borah *et al.*; studied the effect of environment and nutrition on the growth of PHA in cells on a scaffold based on polyhydroxyalkanoates derivative from mutant strain of *Pseudomonas sp.*. In an attempt to enhance PHA quality, naive PHA was blended with biodegradable polymeric materials like polyethylene glycol (PEG), polylactic acid (PLA) etc. to improve the scaffold properties.

They reported that PHA: PEG showed better scaffold prepared supports better cell growth than plastic surface (Borah *et al.*, 2002). In a similar effort Shabna *et al.*; evaluated the growth of cells on a scaffold based on polyhydroxyalkanoates derived from the mutant strain of *Pseudomonas sp.* They reported that PHA: PEG showed better scaffolding than other samples. It also stated PHA: PEG scaffold prepared supports better cell growth than plastic surface (Shabna *et al.*, 2013). In 2012 Singha *et al.*; studied the production of PHA copolymer by *Bacillus thuringiensis*. strain EGU45. They produced the new copolymers NP along with hydrogen and PHB using synthetic media (Singh *et al.*, 2013).

II. MATERIALS AND METHODS

Site description and sample collection

Sewage and sewage sludge samples were into polycarbonate bottles. They were then transported to the laboratory and processed within 48 hours.



Isolation of bacteria from sewage samples

Glass wares and the growth media were sterilized prior to work by autoclaving for 15 to 20 minutes @ 121° C° (15 lbs). Used cultures/ biohazards were autoclaving for 1 hour @ 121° C (15 lbs) for decontamination.

Serial dilution

The objective of this operation is to inoculate a series of tubes with microbial suspension so dilute that the probability of introducing even one individual in a given tube is very small. 100 µl from the prepared dilutions (10⁻², 10⁻³, and 10⁻⁴) were used for inoculating growth mediums.

Growth media and inoculation

The microbes were inoculated on Luria agar media (LA) also known as the LB Agar consisting of Luria Bertani (LB) broth (Himedia; Cat No. LQ118-50X10ML) supplemented with agar (Himedia; Cat No. RM026) and the E2 Minimal media were prepared to culture the samples. LB media was used as it permits fast growth and good growth yields for many species. The E2 minimal media is rich in salt and allows the change of the carbohydrate source in accordance to the preference of the target inoculum. It has been used to mimics essential nutrient stress which induces production of PHA.

The plates were inoculated using the spread plate technique

Colony characterization

Colony Morphology

Colony morphology is important attribute as different species of bacterium produce very different colonies thus facilitating characterization. Morphological characterization was done using the following parameters: form, size, color, margin and consistency. The colonies were further characterized by Gram staining

Identification of PHA inclusions in vivo

Sudan Black B staining

Using a sterile loop (Himedia Metalloop; Cat No. LA012), we spread the culture on a clean, grease free slide. The smear is heat fixed and a few drops of Sudan Black B solution are spread on the fixed preparation. The ethanol in the stain evaporates in about 5-10 minutes. The excess liquid was carefully drawn off using a filter paper. The slide was then immersed in xylene until it was completely decolorized. The slide with counterstained with safranin (Himedia 0.5% w/v; Cat No. S027- 125ML). After 10 seconds, the slide was washed with running tap water and air dried and visualized under oil immersion.

Nile Blue A staining

We incubate heat fixed smears with solution 1, 1% w/v aqueous solution of Nile blue A (Himedia, RM394-10G) at 55°C for 10 minutes, washed with water to remove excess stain and add solution 2, 8% (w/v) acetic acid (Sigma, A6283-100ML) for 1 minute. The slide was washed with water and blotted dry and a cover slip was applied. The slides were viewed using a 40X epi fluorescence microscopy using a green filter providing an excitation wavelength of 460 nm. PHB granules fluoresce strongly red/bright orange.

Genotypic analysis of bacterial isolates

Bacterial DNA were isolated from the cultures and analyzed for the following. DNA was directly used for the subsequent experiments or stored at -20°C. Qualitative analysis of the samples were done by preparing a 2% agarose gel and a 8% polyacrylamide gel.

Visualization and analysis

After electrophoresis, the bands were visualized using the Carestream gel documentation system (Rochester, NY) and the images were documented using the Image Station 4000 R software. Preliminary work was also carried out using a UV trans-illuminator.

PCR

A premixed master mix (Promega GoTaq green, M7122,) was used following the recommended specifications. 1µl of gDNA sample was taken in each of the PCR vials (Corning Axygen 0.5 ml flat cap, AXY-PCR-05-L-C) and of master mix is added to it and mixed properly. PCR reactions are carried out in a thermal cycler (Eppendorf Mastercycler). The amplicons were then resolved using agarose/PAGE gel electrophoresis.

Ribosomal Intergenic Spacer Analysis (RISA)

2 µl of genomic DNA from the 6 samples was added to each reaction tube and amplified following these conditions. A No template control (NTC) was included in all reactions. gDNA was replaced by equivalent amount of nuclease free water (Promega). The amplicons were resolved in both PAGE and agarose gels.

16S ribosomal DNA amplification

2 µl of genomic DNA from the 6 samples was added to each reaction tube and amplified following these conditions. A No template control (NTC) was included in all reactions. Genomic DNA was replaced by equivalent amount of nuclease free water (Promega). The amplicons was resolved in a 2% agarose gel.

DNA sequencing and phylogenetic analysis

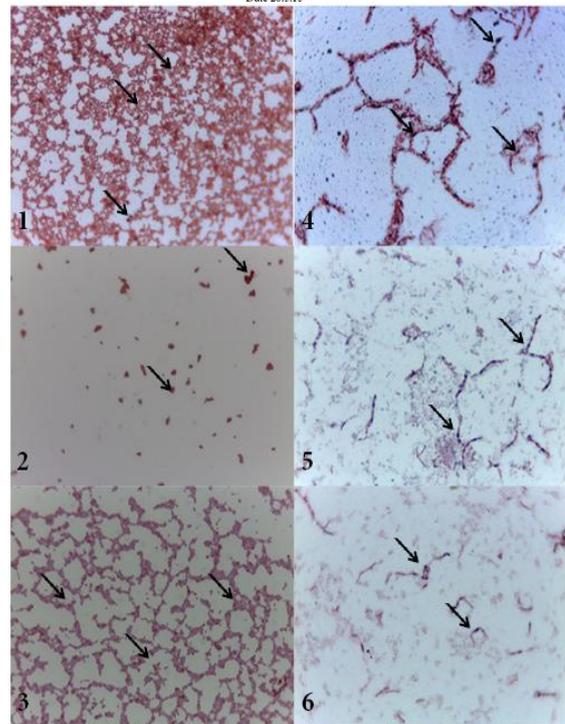
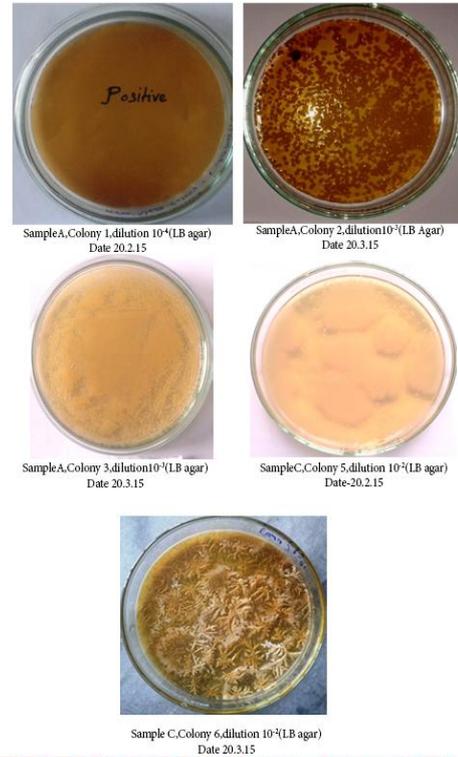
The sequencing of purified 16S r DNA fragment was performed with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on a ABI Prism377 DNA Sequencer (Applied Biosystem) at a commercial company (SciGenom Sequencing Facility, India).

The sequences of 16S r DNA genes were uploaded in the (Ribosomal Database Project) RDP database.

III. RESULTS ANALYSIS

Sudan Positive Isolates

Sudan Black B staining was done for all samples in slides and observed under the microscope. Out of 20 colonies isolated and stained, 6 positive isolates were found which showed bluish black staining. This was a preliminary test for identification of PHA accumulating bacterial colonies. These colonies were subcultured and preserved temporarily under -20°C for future use

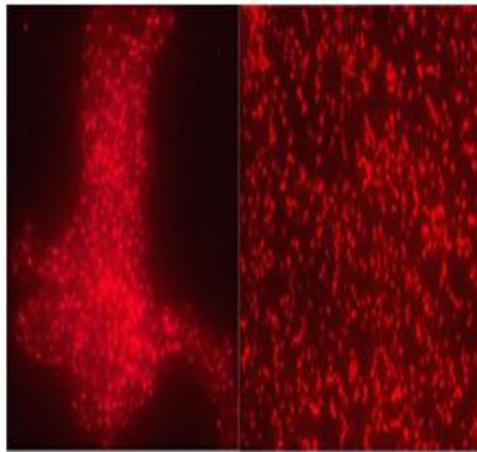


Bacterial cells after 48-72 hours incubation under 40X magnification. Lipid inclusions were coloured with sudan black B (Col. 1, 2, 3, 4, 5 and 6). Staining and detection of cells was performed with safranin

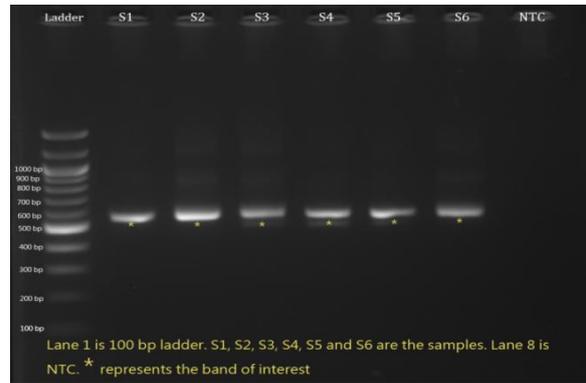
Staining with Nile BlueA

The 6 positive isolates were further stained with Nile blue A, which is the confirmatory test for identifying PHA production. An orange fluorescence was observed for all the Sudan positive isolates after staining.

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Colony 1 Nile staining Colony 3 Nile staining



Phylogenetic analysis

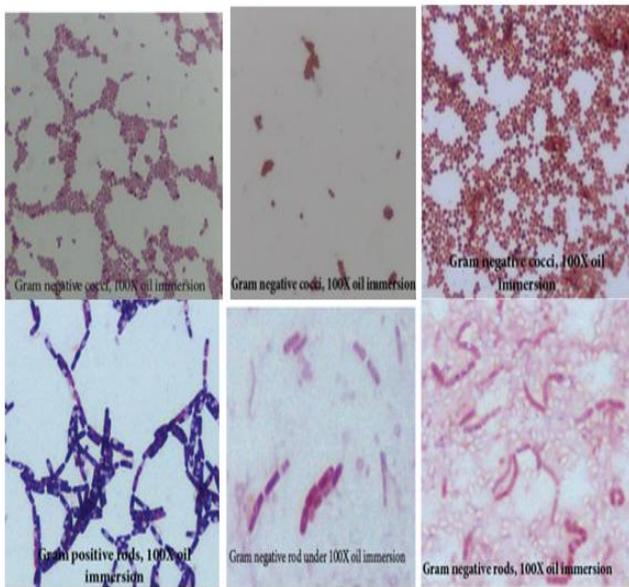
Alignment of sequence with reference sequence database
We attempted sequencing of all the six 16S rDNA amplicons, however only three gave satisfactory results. Sample 1, sample 3 and sample 6 were lost during trimming as their sizes were below 200 bp threshold. The rest of the 3 sequences were uploaded to the RNA Database Project (RDP release 11, update 4:: May 26, 2015). After uploading, the sequences were available under the myRDP profile. After analyzing with “Seqmatch” we got the following identities of our isolates

Results for/sample.5_8F

DOMAIN:	Bacteria
PHYLUM:	Firmicutes
CLASS:	Bacilli
ORDER:	Bacillales
FAMILY:	Bacillaceae
GENUS:	Bacillus

S00005079	0.9950.9861395	Bacillusthurin
S00005826	0.9950.9861393	Bacillus cereu
S00006577	0.9950.9861395	Bacillusthurin
S00008107	0.9950.9861396	Bacillusthurin
S00008663	0.9950.9861397	Bacillusanthr
S00011557	0.9950.9861358	Bacillus sp.;
S00012518	0.9950.9861397	Bacillusanthr
S003807776	0.9950.9881383	Bacilluscereu
S004450793	0.9950.9881386	Bacilluscereu
S004168652		
0.9950.9881294		uncultured bacterium;
nck214h12c1;		KF095870

Colony characteristics of selected colonies on LB agar



DNA extractions from mixed bacterial culture

The image below depicts extracted DNA as seen after resolving in a 1.5% agarose gel

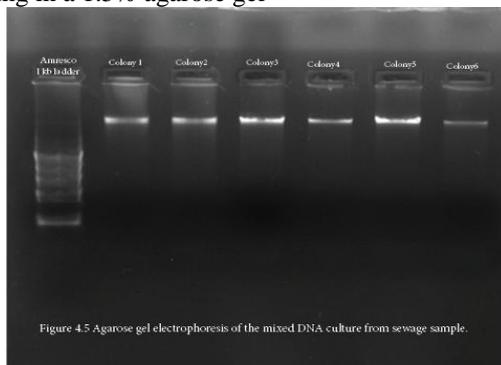


Figure 4.5 Agarose gel electrophoresis of the mixed DNA culture from sewage sample.

Agarose Gel electrophoresis of 16s rDNA amplification

The 430 bp 16S rDNA amplicon was resolved in a 2% agarose gel against a 100 bp DNA ladder. The bands for each isolate can be seen in the images below.

Resultsfor|sample.4_8F

DOMAIN:	Bacteria
PHYLUM:	Firmicutes
CLASS:	Bacilli
ORDER:	Bacillales
FAMILY:	Bacillaceae
GENUS:	Bacillus
S000005079	0.991 0.988 1395 Bacillus thuringiensis; WS 2617; Z84585
S000005826	0.991 0.988 1393 Bacillus cereus; ATCC 27877; Z84581
S000006577	0.991 0.988 1395 Bacillus thuringiensis; WS 2626; Z84588
S000008107	0.991 0.988 1396 Bacillus thuringiensis; WS 2618; Z84586
S000008663	0.991 0.988 1397 Bacillus anthracis; Sterne; AF290552
S000011557	0.991 0.988 1358 Bacillus sp.; JJ#1; Y15466
S000012518	0.991 0.988 1397 Bacillus anthracis; Vollum; AF290553
S003807776	0.991 0.990 1383 Bacillus cereus; CH-13; KF151160
S004450793	0.991 0.990 1386 Bacillus cereus; AUChE413; KM598765 unclassified_Bacillales
S004168652	0.991 0.990 1294 uncultured bacterium; nck214h12c1; KF095870

Resultsfor|sample.2_8F

DOMAIN:	Bacteria
PHYLUM:	Firmicutes
CLASS:	Bacilli
ORDER:	Bacillales
FAMILY:	Bacillaceae
GENUS:	Bacillus
S001745448	0.955 0.780 1432 Bacillus sp. EWF34; GU120655
S001935541	0.955 0.780 1399 uncultured bacterium; 1G10; GU359077
S002199758	0.955 0.780 1402 uncultured Bacillus sp.; Filt.29;
HM152617	
S002199821	0.955 0.780 1431 uncultured Bacillus sp.; Filt.93;
HM152680	
S002199836	0.955 0.780 1435 uncultured Bacillus sp.; Filt.108;
HM152695	
S002226854	0.955 0.780 1223 Bacillus cereus; SP7; HM188452
S002231093	0.958 0.800 1232 Bacillus sp. DB07(2010); HM566809
S002231463	0.955 0.780 1223 Bacillus cereus; HM573311
S002234931	0.955 0.780 1435 Bacillus cereus; TAT2-12; HQ236069
S002268450	0.955 0.780 1308 uncultured bacterium; nby549h07c1;
HM835821	

Phylogenetic tree

All the above (29) near neighbour isolates selected by Seqmatch along with the 3 samples were aligned and a phylogenetic tree was grown using MEGA.

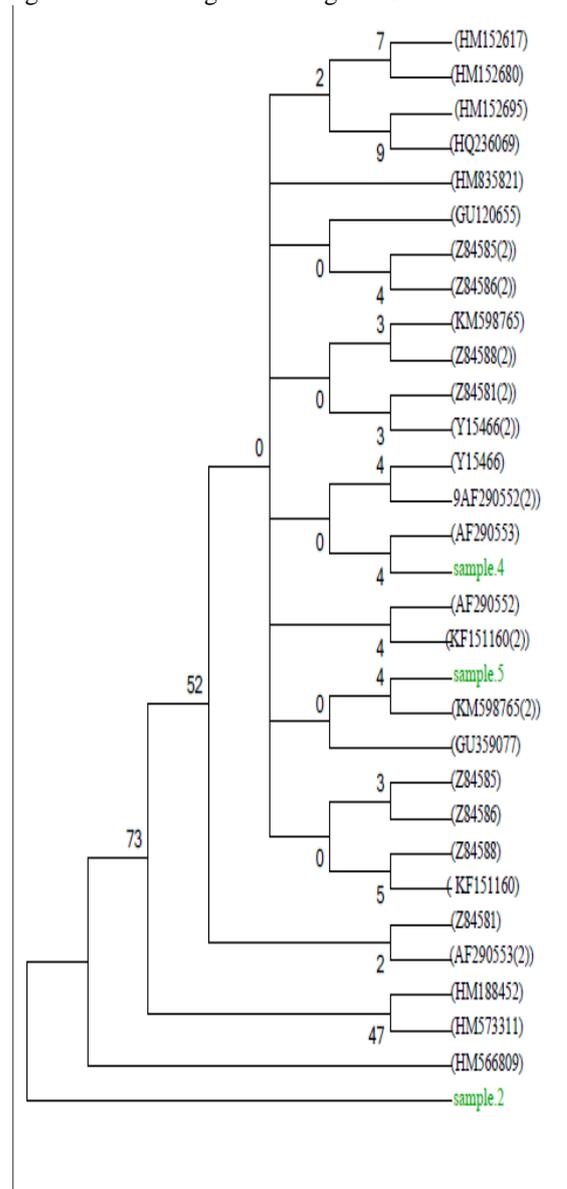


Figure 3.7.2 Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [6]. The tree with the highest log likelihood (-514.6627) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0500)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 25.8333% sites).

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The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 303 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [21].

IV. CONCLUSION

We had isolated 20 colonies from different sewage samples, out of which 6 colonies were found to be positive for PHA accumulation. However after sequencing of the 16S rDNA, convincing results were obtained for 3 samples only. Phylogenetic analysis of the 3 samples indicated that they all belong to the genus *Bacillus*. One of our samples (sample 2) appears to be a novel strain and thus needs further characterization. Our observations correlate with several previous reports wherein the production of PHA has been reported. For instance *Bacillus* sp. CFR67 accumulating PHA using corn starch and other agro-industrial waste as a substrate was reported by Shamala *et al.* The production of PHA using *Bacillus* species was also reported by Kumar *et al.* *Bacillus* as emerged as a robust organism that can withstand adverse environmental conditions. Other ideal features that could make *Bacillus* ideal for industrial production of PHA include facts such as possession of single membrane which could ease downstream processing as PHA granules are accumulated intracellularly, fully sequenced genome and natural competency which could facilitate strain improvement efforts and finally *Bacillus* are not major pathogens to humans. Growth parameters such as temperature (25-32°C) and pH(6.8-7.2) were favoured as our aim was to isolate bacteria which had increased biological activity without the need of specialized conditions. Although our study doesn't document PHA accumulation scientifically, during staining procedures we observed that bacterial accumulation of PHA increases from 48 to 72 hours of incubation, and declines on further incubation. We corroborate our observations to the microscopic method of Ostle and Holt(1982) wherein the intensity of fluorescence changed with incubation period.

PHA holds the key to a greener planet. Although our study suffered from several limitations we believe that we can contribute significantly towards the development of the technology in the days to come.

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AUTHOR PROFILE



Jasmine Chetia, daughter of Sri Anil Chetia and Mrs Rupa Chetia, resident of Golaghat, Assam. She did Msc in Biotechnology from Gauhati University in 2015. Presently she has been working as Assistant Professor (Contractual) in the department of Botany, Sonari College, Sonari, Assam-785690. She has published 3 papers in various ugc approved journals and had presented 2 national seminars. She has undergone two months summer training at RMRC, Dibrugarh. A project entitled "To learn the different techniques involved in biomedical research including Sample processing, Nucleic acid extraction, Polymerase Chain Reaction, ELISA and also Cell culture Techniques" under the guidance of Dr. D. Biswas (Scientist – E).