ISOLATION, SCREENING AND EXTRACTION OF POLYHYDROXYBUTYRATE(PHB) PRODUCING BACTERIA FROM SOIL SAMPLE

Ruchita R[1], Vismaya N Kumar[2], Nair Sreecha Chandran[3], Sonia John[4]
Project Assistant,
Cepci Laboratory and Research Institute,
Mundakkal, Kollam.

Abstract

Plastic and polyesters have wide spread use in today's society because of their processability and amenability in providing a large variety of cost effective products that help to enhance comfort and quality of life. Synthesis of plastic and synthetic polymers from non renewable resources and after intended use ,resulting into environmental pollution.Therefore the value in developing biodegradable plastics such as poly hydroxyl butyrate (PHB), which consist of desirable chemical and physical properties similar to conventional synthetic plastics and are environment friendly too.In the present study an attempt was made to isolate efficient PHB producing bacteria from highly polluted soil.From the different colonies a single type of bacteria were isolated and found to be PHB positive based on the viable colony staining method using Sudan black B and the organism identified as B.cereus. Then Bacillus cereus found to be quite efficient PHB producer and exhibiting a potential for their utilization in commercial PHB production.

Key words:Biodegradable plastic , PHB production ,Polyhydroxylalkanotes,polyhydroxy butyrate.

1.INTRODUCTION

Two kind of plastic are widely used today which include petroleum based plastics and biodegradable plastics. The development of petroleum based plastic solely dependent on fossil fuels and soil petrol is consumed is such a high levels if may lead to its depletion in coming years. The development of biodegradable plastics has helped researches to overcome the drawbacks of petroleum based plastics. The replacement of synthetic plastic with biodegradable plastic has been found to reduce the consumption of fossil fuels. Biodegradable plastics solve the problems caused by non –biodegradable plastics and also help to overcome pollution problems. Biodegradable plastic can be divided into photodegradable,semidegradable and completely biodegradable plastics.

Poly hydroxylalkanotes(PHAs) are considered to be strong candidates for biodegradable plastic as their material properties are similar to various synthetic plastics.PHA are polyesters accumulated by various bacteria under unbalanced growth conditions. PHA bacteria production which influenced by types of microorganisms,media ingredients,fermentation condition etc.PHB is a polyhyroxyalkonate , a polymer of polyesters. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl co A. Latter compound is then used as momomer to polymerize PHB. PHA granules are the recoverd by disrupting the cells.*Bacillus,Pseudomonas* species can able to degrade the PHB.

PHB are used for making packaging items ,disposable items and biomedical devices etc.The biomedical devices include sutures ,repair devices,repair patches,slings,cardiovascular patches, orthopedic pins and stents ,bone marrow scaffolds and regeneration devices.

2.MATERIALS AND METHODS

2.1.Collection of Samples :

Soil sample were collected in sterile plastic sacs from highly polluted area of kollam and serially diluted according to the following procedure ; Arrange sterile test tubes (cotton plugged) in a test tube stand and label with $10^{-1,}$ 10^{-2} till 10^{-7} dilution. Distribute 9ml of sterile saline solution aseptically. With a sterile pipette, aseptically transfer 1ml of bacterial culture to the test tube labelled as 10^{-1} and then mix the tube by vortexing. Transfer 1ml of this suspension from the test tube labelled as 10^{-2}. Each time the transfer is done with a fresh pipette. Then mix the tube 10^{-2}. Repeat this kind of serial dilutions from one to the next tube till the last tube (10^{-7} dilutions). For the isolation of organisms, 0.1ml of each dilution was plated on a nutrient rich medium by spread plate method for the propagation of microbial growth. The plates were incubated at 37^0C for 24 hour.

2*.2.Maintenance of Bacterial Cultures :*

1.3g Nutrient broth in 100ml distilled water was prepared and incubated at 37^oC for 24 hours. Isolated colonies(serially dialuted) were selected and purified by repeated Zig-Zag and Quadrant streaking on agar plates.

2.3.Screening of the PHB accumulating bacterial strain :

The isolated colonies were screened for PHB production by Sudan Black B Staining and ranked based on the magnitude of staining according to Nandini *et al* and Burdon. Prepare thin smear on microscope slide and thoroughly air dry. Do not heat fix.Stain with Sudan black B solution and let it stand for 10-15 minutes. Add more stain if the slide starts to dry out. Wash the slide with distilled water and counter stain with safranin for 10 seconds. Wash with distilled water and blot dry with tissue paper. Examine the slide under oil immersion microscope for PHB granules. Organisms shows positive in black and shows negative in yellow brown.

2.4.Identification of bacteria by Gram staining:

The bacterial isolates were gram stained to determine the gram nature. A thin bacterial smear was made on a glass slide and heat fixed. Apply a drop of crystal violet stain to the smear and wait for 2-3 minutes. Rinse the slide with distilled water. The smear is treated with grams iodine and wait for 1-2 minutes. Rinse the slide with distilled water. Add two drops of 95% ethanol or 100% acetone to the slide and decolourise it (1-2 seconds). Rinse the slide with distilled water. Add few drops of safranin and wait for 45seconds. Rinse the slide with distilled water and blot dried. Then examined under microscope.

2.5. Extraction of PHB :

PHBs were recovered from the cell mass by sodium hypochlorate method outlined by Rewate and Mavinkuve,(2002). Selected cultures were grown in Erlenmeyer flasks containing 50ml of the medium. A simplified media containing only 1.0%(w/v) glucose, 0.5%(w/v)peptone, and 0.25%(w/v) NaCl as used for the production (Aarthi et al, 2011). The cultures were incubated in a shaker at 37^0C for 48 hours at 150 rpm . After incubation, these cultures were taken and centrifuged at 4000rpm for 30 minutes. The supernatant was discarded and the pellet was treated with sodium hypochlorate equal to the original volume of the medium and the mixture was incubated at 37^0C for 1 hour. The incubation mixture was subjected to centrifugation at 4000rpm for 30 minutes and was washed with water, acetone and alcohol respectively. These pellets were dissolved in 5ml of chloroform which then evaporated by pouring the mixture on the sterile glass trays .

2.6. Identification of bacteria by 16s rRNAsequencing :

The selected strain of bacteria after preliminary screening through staining techniques were subjected to genotype characterization using 16s rRNA partial gene sequencing and sequence analysis using bioinformatics tools for identification upto species level.

2.7. Isolation of genomic DNA :

1.5 ml over night culture of the isolate was transferred to a 1.5 ml microfuge tube and centrifuged at 12000rpm for 10 minutes and supernatant was decanted. The cells were resuspended in 400 ml TE buffer, vortexed and 50 ml 10% SDS, 50 ml proteinaseK (20mg ml^{-1} in TE) were added and incubated for 1 hour at 37^0C. The contents were pipetted several times using micropipette. This was extracted twice with 500 ml phenol: chloroform(1:1), and twice with 500 ml chloroform by centrifuging the sample each time at 12000rpm for 10 minutes. The nucleic acids were precipitated by adding 25 ml 5M NaCl and 1 ml 95% ethanol and centrifuged for 10 minutes at 12000rpm. The supernatant was discarded and the pellet was dried (Babu et al., 2004).

2.8. Purification of the isolated DNA :

The pellet was resuspended in 100ml TE buffer, 5ml ribonuclease (5mg ml^{-1} in TE) was added and incubated at 37^0C for 30 minutes. The DNA was precipitated with 40ml 5M ammonium acetate and 250ml isopropanol and incubated at room temperature for 5 minutes. The pellet was washed twice with 70% ethanol followed by centrifuging at 12000rpm for 10 minutes at the end of each step. Pellet was dried until the whole alcohol gets evaporated and dissolved in 50ml TE buffer and was then stored in -20^0C (Babu et al., 2004).

2.9. Quality check of DNA :

The quality of DNA isolated was checked using agarose gel electrophoresis method as suggested by Sambrook et al.,(1989). 1 litre of electrophoretic buffer (1X TAE buffer) was prepared from 50 X stock solution. O.6g of agarose was dissolved in 60ml of 1X TAE buffer (1% agarose) by boiling at 60^0C in a microwave oven. It was allowed to cool to 40^0C and to this 0.5micro litre ethidium bromide (EtBr) was added carefully using a micropipette. It was casted on a gel tray with well formers (comb) bound on both sides with cellophane tape and sample was loaded. 5micro litre of 1 kb DNA ladder (NE biolabs) was used as the marker and electrophoresed at 60V for 1hour. The agarose gel was taken from the apparatus and placed in a UV transilluminator and documented through Gel Doc System (Alpha imager, USA).

2.10. PCR amplification of 16S rRNA gene :

Bacterial 16S rDNA was amplified from the extracted genomic DNA by using the following 16S rDNAprimers : forward 8F primer 5'AGAGTTTGATCCTGGCTCAG 3' and reverse1492R primer 5'CGGCTACCTTGTTACGACTT3' Babu et al.,(2004). The reaction was carried out in Eppondrof thermal cycler. Polymerase chain reaction was performed with 25micro litre reaction mixture containg 4microlitre of DNA as template, each primer at a volume of 1microlitre, 2.5microlitre Taq buffer, and deoxynucleotide triphosphate at a volume of 1microlitre, as well as 0.5microlitre of Taq polymerase and made up to required amount by sterile water. After the initial denaturation for 4.5 minutes at 95˚C, there were 40 cycles consisting of denaturation at 95˚C for 1 minute, annealing at 55˚C for 1 minute,and extension at 72˚C for 2 minutes and then a final extension step consisting of 5 minutes at 72˚C ; Thermal cycler (Eppendrof , Germany) was used. The amplification of 16S rDNA was confirmed by running the amplification product in 1% agarose gel electrophoresis in 1X TAE with 100bp marker (NE Biolabs) and photographed with gel documentation system(Sygene, USA).

2.11. Purification of 16S rRNAamplicon :

The amplified 1500-bp product was cut using a gel cutter, eluted and purified using the gel extraction kit (QIAGEN). The DNA bands amplified using 8F and 1429R primers were excised from the agarose gel using a gel cutter. An empty eppendrof tube was weighed using an electronic precision balance, the gel slice was put into it and the weight was again taken. Then 3 volumes of buffer QG were added to 1 volume of gel. This was incubated at 500˚C for 10 minutes. The tube was then vortexed to help dissolve the gel. One gel volume of isopropanol was added to the sample mix and applied to the QIA quick column follwec by centrifugation for 1 minute. The flow through was discarded and the QIA quick column was placed back in the same collection tube. The column was then washed with 0.75ml of buffer PE and centrifuged for 1 minute. The flow through

was discarded and the QIA quick column was centrifuged for an additional 1 minute at 10000rpm. The column was then placed in clean 1.5ml vial.50micro litre of sterile water was added to the centre of QIA quick membrane and centrifuged for 1 minute. This was run in 1% agarose gel with 100bp marker in 1X TAE. Finally 50micro litre of the eluted 16S rDNA was stored in the refrigerator.

2.12. Checking for the re-amplification of the eluted product :

The eluted product was subjected to re-amplification using the same 16S rDNA primers: forward 8F primer 5'AGAGTTTGATCCTGGCTCAG 3' and reverse 1492R primer 5'CGGCTACCTTGTTACGTTACGACTT 3' (Babu et al., 2004). Polymerase chain reaction as performed with 25μL reaction mixture containing 4μL of eluted product as the template, each primer at a volume of 1μL, as well a 0.5μL of Taq polymerase and made up to required amount by sterile water. The amplification was done with the same cycling programme. After the initial denaturation for 4.5 minutes at 95^0C there were 40 cycles consisting of denaturation at 95^0C for 1 minute, annealing at 55^0C for 1 minute, and extension at 72^0C; Thermal cycler (eppendorf,Germany) was used. The amplification of 16S rDNA as confirmed by running the amplification product in 1% agarose gel electrophoresis in 1X TAE with 100bp marker (NE Biolabs)

2.13. Sequencing and identification of the isolate :

The eluted product along with the primers: forward 8F primer 5'AGAGTTTGATCCTGGCTCAG3' and reverse 1492R primer 5'CGGCTACCTTGTTACGACTT 3' were sequenced at RGCB,Thiruvanthapuram for sequencing according to their specifications. The sequenced strands were edited using the BIOEDIT software. The nucleotide sequence were compared with those in the NCBI database using the Basic Local Alignment Search Tool (BLAST). The sequence obtained for the bacterial isolate was aligned with each other by using Clustal W multiple alignment programme of Bio-Edit software.

3.RESULT AND DISCUSSION

3.1.Isolation of bacteria :

Microorganisms was isolated (serial dialution;10^{-1} to10^{-7}) from polluted soil of kollam District. The surface colony morphology shows variations. Large group of colonies have moist surface. Similarly it is observed that slight variations in colony shape and colour. Mostly circular shaped isolates are observed and in the case of colony colour, white colourobserved.There for the large white coloured single colonies are selected for the further studies.

Even though Several studies have shown that a number of bacterial strains, both Gram positive and Gram negative, produce PHB, the sheer diversity of the microbial world calls for continuous screening and identification of bacteria capable of utilizing cheap nutrient sources for production of large quantities of PHB *Bacillus sp.* were isolated from groundnut plant rhizosphere and subjected to morphophysiological and biochemical characterization. Isolation and screening of potential bacterial strains from natural environment and improvisation of bioprocess parameters has been an extensive area of research in the field of PHB production (Israni & Shivakumar 2015).

3.2.Pure culture formation

Pure colonies were obtained through repeated Zig-Zag streaking (Isolated colonies from serial dialution 10^{-5}, 10^{-6}, 10^{-7}) and it is used for further studies.

3.3. Screening of isolates for PHB production :

3.4. Sudan black-B staining :

Black colour granules were taken as positive result. The appearance of black coloured granules in the cell indicates PHB production (FIG 6.1). The bacteria positive for PHB production were selected by granules under a fluorescence microscope, bacterial culture showing substantial fluorescence were selected for further study.

Phenotypic detection method like Sudan Black-B staining (Schlegel *et al*., 1970) has been widely used to screen micrbes accumulating PHAs. PHB inclusion stained with Sudan Black indicated that they are lipoidal in nature (Williamson et al., 1958). Hartman, (1940) was the first to suggest the use of Sudan Black as the bacterial stain. It is non-specific to PHA as it stains other lipid bodies. Subsequently Burdon et al., 1942 confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by prepairing microscopic slides of bacteria stained with Sudan Black solution and counter stained with Safranin.

3.5.Extraction of PHB :

Sodium Hypochlorite solubilises non- PHA cellular materials and leaves PHA intact. The PHB was separated from other cellular materials by centrifugation with water, acetone and alcohol and finally dissolved in chloroform. Evaporation of the chloroform resulted in the formation of a whitish thin film (FIG 6.2) which indicated PHB production by the selected colonies whereas the colonies without PHB production did not showed film.

3.6. Characterization and identification of the selected bacteria :

3.7.Colony morphology staining :

PHB producing bacterial strains were characterized by gram staining. The isolates were Gram positive and rod shaped (FIG 6.3)

3.8. Identification of bacteria by 16s rDNA :

The selected strain producing high amount of PHB was identified upto species level by partial 16s rDNA sequence analysis. Using the described DNA isolation, purification and amplification conditions with the selected primer, good and clear 1500bp band was obtained. Partial sequencing of this PCR product was done (figure 6.4). Partial 16s rDNA sequences available in the gene bank, which includes numerous public databases by using BLAST search. The BLAST analysis of the sequence showed 99% similarity with *Bacillus cereus.* The 16s rDNA sequence analysis and morphological tests helped to identify the strain as *Bacillus cereus*.

Sequencing of 16s rRNA genes has been carried out by many researchers to identify PHA accumulating strains from various econiches such as soil, activated sludge, marine sediments etc. (Valappil et al., 2007b, Reddy et al., 2009, Arun et al., 2009).

On comparing the partial sequences of 16s rDNA with those in NCBI database, the selected strain showed maximum identity (99% identity) with other B.cereus strain. The nucleotide sequence was deposited in the GeneBank database under accession number JF-760212.

Sequencing of 16s rRNA genes was crucial for careful and unambiguous identification of the environmental isolates (Halami, 2008). The 16s rRNA gene sequence analysis and morophological studies helped the identification of newly isolated PHB producing Bacillus. 99% of similarity value was observed between partial sequences of the 16s rRNA genes of isolate with that of Bacillus species available in the public nucleotide databases of NCBI using the BLAST algorithm.

3.9. Aligned sequence of 16s rDNA :

Aligned sequence closely related to bacillus cereus (figure 6.5)

4.CONCLUSION

The main objective of the present study was the production of biodegradable plastic by using beneficial microorganism to improve environment and welfare of human beings throughout the world.The results have thrown a light to the possibilities of utilizing and proper management of highly polluted soil for different applications. In the near future PHAs will play a major role as biodegradable plastic in a wide range of application and will help to eliminate disposable problems and environmental hazards caused by conventional plastics.

5.REFERENCE

1. Aarthi N and Ramana K V, (2011) , Identification and characterization of polyhydroxybutyrate producing Bacillus cereus and Bacillus mycoides strains, Int J Environ Sei, 1 (5) 744-756.
2. Arun A, Arthi R, Shanmugabalaji V N and Eyini M, (2009), Microbial production of poly-beta-hydroxybutyrate by marine microbes isolates from various marine environments, Bioresour Technol, 100, 2320-2323.
3. Burdon, K. L., Stokes, J. C. and Kimbrough, C. E., (1942)a, Studies of the common aerobic spore-forming Bacilli staining for fat with Sudan Black B- stain. Journal of Bacteriology, 43 : 717-724
4. Halami P M , (2008), Production of polyhydroxyalkanoate from starch by the native isolate Bacillus cereus CFRO6, World J Microbial Biotechnol, 24 ,805-812.
5. Hartman T. L., (1940), The use of Sudan Black B as a bacterial fat stain. Staining Technology, 15: 23-28
6. Israni, N. & Shivakumar, S.(2015). Evaluation of upstream process parameters influencing the growth associated PHA accumulation in Bacillus sp. Ti3. Journal of Scientific and Industrial Research 74: 290-295.
7. Nandini P, Amruta C, Bhavesh P, Pragya R, Priti V, Mital P. Screening of PHB (polyhydroxyalkanoates) producing bacteria from diverse sources. Microb Biotechnol. 2011;36:216–74.
8. Reddy, S.V., Thirumala, M. and Mahmood, S.K. (2009). Production of PHB and P(3HBco-3HV) biopolymers by Bacillus megaterium strain OU303A isolated from municipal sewage sludge. World J. of Microbiol. Biotechnol., 25(3): 391-397. 27.
9. Rewate T and Mavinkurve S, (2002), A Rapid hypochlorate method for extraction of polyhydroxyalkanates from bacterial cells, Ind J Exp Biol, 40 ,924-929.
10. Schlegel H G, Lafferty R and Krauss I,(1991), The isolation of mutants not accumulating polyhydroxybutyric acid, Arch Microbiol,75 (4)283-294.
11. Valappil S P, Boccacccini A R, Bucke C, Roy I, (2007) , Polyhyrdroxyalkanoates in Gram-positive bacteria: insights from the genera Bacillus and Streptomyces Antonic Van Leeuwen Hock, 91,1-17.
12. Williamson D H and Wilinson J F, (1958),The isolation and estimation of polybetahydroxyl inclusions of Bacillus sp, J Gen Microbiol, 19 ,198-203.

AKNOWLEDGMENTS

First of all I would like to thank the almighty for showing his blessings to fulfill my work without any obstacles and for giving strength to complete my work.Then I would like to express my sincere gratitude to our guide Ms Prabhakumari .C, Cashew Export Promotion Council of India, Kollam, for her constant support, guidance, Concern and inspiration throughout my project. And I would also like to express our gratitude to Mrs.Nair

Sreecha Chandran,Mrs Soniya Ms and Vismaya N kumar ,phd scholars, Cashew Export Promotion Council of India, for their constant support, guidance, and co-operative throughout my work. Lastly, I owe my parents and friends for their moral support, encouragement and co-operation throughout my work.

6.LEGENDS

Figure 6.1; sudan black staining method – the PHB accumulating strain appear in black colour

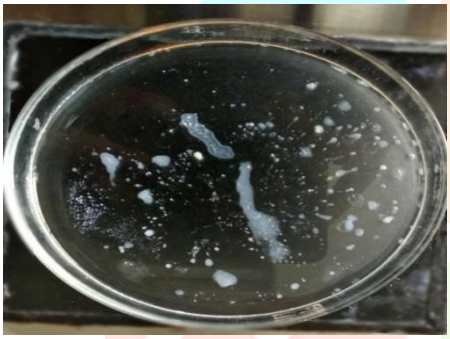

Figure 6.2; PHB extracted by sodium hypochlorite method

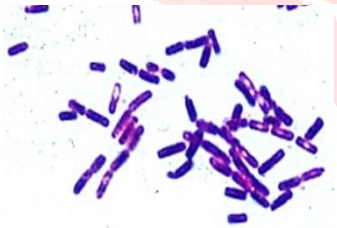

Figure 6.3; gram staining of colonies shows rod shaped and violet coloured, gram positive bacteria.

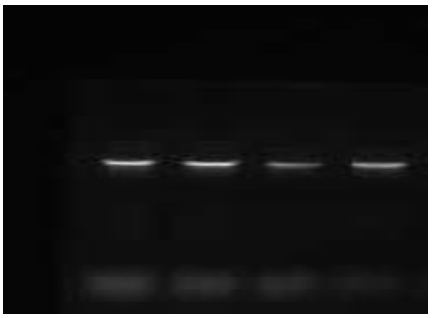

Figure 6.4;Isolated DNA sample from selected culture on 1%agarose gel.

TTAGCGCGGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAA
ACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTG
TCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTTATGTAACGGCTCACCAAGGCAACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGGATGAGACACGGCCCAGACTCCTACG
GGAGCGAGCACTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGG
CACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATG
TGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGA
AAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGC
GACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGT
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCAT
GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTT
AGTTGCCATCATTAACGAGCGCAACCCGGGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACC
TGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATC
TCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCTTACATTGAAGCTGGAATCGCTAG
TAATCGCG

Figure 6.5;Aligned sequence of 16s rDNA