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Molecular Identification of PHB Producing Bacteria Isolated From Marine Water Habitat.

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Abstract:

In the day to day life increases the use of plastic these causing hazard effects to environment to reduce these problem using biodegradable plastic. Bacterial poly-β-hydroxybutyrate (PHB) is a natural, biodegradable polymer, which is accumulated in the cells as an energy reserve material due to depletion of nitrogen or phosphorous in the presence of excess carbon source. This polymer is excellent alternative to the non-degradable petroleum-based plastics. In this study, isolated and identified halophilic PHB producers. The 4 PHB synthesizing microbial strain was isolated from Mumbai Juhu Beach Maharashtra India and they screened by Sudan black B staining method. All four strains were the *MA*1 and *NA2* shows high amount of PHB these two isolates were further biochemically and molecularly identified by National Chemical Laboratory Pune (CSIR) MS India. Analysis of 16s rRNA gene sequence of the two strains revealed 99.86% and 99.51% similarity to that of *Halomonas salifodinae* and *Bacillus licheniformis*, respectively. The pure cultures of isolates were preserved on slant for further use.

Keyword: PHB, Marine water, Bioplastics, *Halomonas salifodinae*, *Bacillus licheniformis.*

Introduction:

Plastics have multipurpose characters of strength, lightness, durability and resistance to degradation. They become an important product in the daily life and have replaced glass and paper in packaging. However, its accumulation becomes one of the greatest problem facing modern civilization due to its degradation resistance (*Khanna and Srivastava 2005*). The alternative solution was the development of eco-friendly and biodegradable biopolymer materials

(*Khardenavis et al. 2007*). One of these biopolymers is polyhydroxyalkanoates (PHAs) which comprise a class of polyesters that are synthesized by most prokaryotes as an insoluble inclusion in their cytoplasm to levels as high as 90% of the cell dry weight (*Madison and Huisman 1999;*

Potter and Steinbuchel 2006). They have mechanical properties similar to polypropylene or polyethylene and can be extruded, molded, spun into fibers, made into films and used to make heteropolymers with other synthetic polymers. They are also completely degraded to water and

carbon dioxide under aerobic conditions and to methane under anaerobic conditions by micro-organisms in soil, sea, lake water and sewage (*Khanna and Srivastava 2005*). The PHA family includes several polymeric esters like polyhydroxybutyrates, polyhydroxybutyrate co hydroxyvalerates (PHBV), polyhydroxybutyrate co-hydroxyhexanoate (PHBHx) and polyhydroxybutyrate cohydroxyoctonoate (PHBO). However, poly3-hydroxybutyric acid (PHB) is the most common natural microbial PHA (*Singh and Parmar 2011*).

Among different types of PHAs reported, only two forms i.e., PHB homopolymer and 3HB-3HV (-3-hydroxybutyrate 3hydroxyvalerate) copolymer are commercially produced. PHB is the best characterized PHA found to be produced by several micro-organisms (Madison and Huisman 1999). It was first discovered in bacteria in 1926 by Lemoigne as a unique intracellular polymer accumulated under stress conditions but in presence of excess carbon source. The numerous bacteria have been reported to be PHB producers and a few of them are being used in industrial production of PHB, but, none of them have been found to be capable of economizing the commercialization of large-scale PHB production. Therefore, there's still a great need, as well as a scope, for the isolation of more efficient novel PHB producing isolates. There are still several PHB producing isolates which are yet to be identified and characterized.

The present study focuses on molecular identification (16s r-RNA sequencing) of two such high PHB producing strains, isolated from marine water sample Juhu beach, Mumbai, India. The present study involved the identification and molecular characterization of two PHB positive bacterial isolates (*MA1 and NA2*) from National Chemical Laboratory Pune, Maharashtra India (CSIR). The isolated organisms are growing on 15% salt concentration, these two isolates had been isolated on nutrient agar and Marine agar by making serial dilutions of the collected marine water samples, and were purified and preserved on nutrient agar slants for further use. Preliminary screening for PHB production was carried out using Sudan Black B dye. (*Lathwal et al.,2015*).

Material and Methods:

Collection of Sample

Collected a marine water sample from Mumbai juhu beach, India. Sample was collected in sterile plastic bottle, it transported to the laboratory aseptically and stored at 4°C temperature in refrigerator for further use.(*Fatma Karray et. al 2021*)

Enrichment of sample

Take 1 ml of sample marine water and add in 100 ml of nutrient broth for enriched the sample. It helps to growing and reproducing of microorganisms. Prepare the serial dilution is to get dilutions 10^{-1} to10^{-3}. For the isolation of organisms, 0.1ml of each dilution was plated onto a nutrient rich selective media's for isolation

of microbial colonies with different characteristic. The inoculated plates were incubated at 37°C for 5-6 days *(Mohsin Azhar et.al 2014*)

Testing of salt tolerance capacity

Take 10^{-3} diluted sample it was streaking on Nutrient agar with different concentration of NaCl. Such as, 5%, 10%, 15% and 20%. Such various concentration of salt cheeked the salt tolerant capacity of bacteria in isolated marine water sample.

Isolation of Halophiles:

The higher PHB producing organisms were isolated using two different selective media's such as Marine and Nutrient agar with 15% salt concentration.

Screening of PHB Producers using Sudan Black B staining:

Detection for PHB production was employed by using lipophilic stain Sudan Black B. Stain was prepared by dissolution of 0.3 gm powdered stain in 100 ml of 70% ethanol. For microscopic studies, smears of colonies were heat-fixed on clean, grease-free glass slides, followed by staining with 0.3% solution of the Sudan Black B. After leaving the slides undisturbed for 25-30 minutes, immersion in xylene and counterstaining with safranin (0.5% w/v in sterile distilled water) was performed for 5 to 10 minutes. Cells appearing blue-black granules with pink cytoplasm was observed under 100X oil immersion objective of light or compound microscope were accredited as PHB positive strains. The pure culture of PHB positive strains were preserved on slant for further analysis (*Dr. R.C. Dubey and Dr. D. K. Maheshwari*).

Phenotypic identification of PHB positive bacterial isolates

The traditional identification of bacterial isolates is based upon studies of their phenotypic characteristics. The two isolates *MA1 and NA2* were therefore subjected to a preliminary phenotypic identification by conducting different standard biochemical tests (*Harish kumar et. al 2013*).

Molecular identification of the PHB positive bacterial isolates

The identification of bacterial isolates based upon conventional techniques, most commonly involving the standard biochemical tests has been observed to be not as accurate as the identification based upon genotypic methods. Therefore, in the present study, after a preliminary phenotypic identification, a final confirmatory identification of the two PHB positive isolates was carried out by using molecular techniques involving 16S rRNA gene sequence homology studies. The step-wise details of molecular identification have been presented in the succeeding.

Procedure from DNA extraction to sequencing: Genomic DNA was extracted by using HiPurA kit and checked on 1% agarose gel electrophoresis.PCR amplification of bacterial specific 16s rRNA gene (1500 bp) was carried out and P CR products were detected by staining with GelRed Nucleic Acid Gel Stain on 1% agarose electrophoresis gel in (1X) TBE buffer and visualized under UV transilluminator. PCR product was purified using purification Kit. Sequencing reactions were run on a 3500xL Genetic Analyzer (Applied Biosystems, USA). The program compares nucleotide or protein sequences to sequence databases and

calculates the statistical significance of matches (*Gertz, 2005*). The BLAST algorithms used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

Result and Discussion:

The present study was designed to identify halophilic PHB producing isolates which had been isolated from juhu beach, Mumbai, Maharashtra, India and to attempt biochemical and molecular identification of the PHB producing halophilic isolates.

Each five isolates are isolated from two different selective media's but in these isolates 2 organisms was showing PHB granules inside their cytoplasm. Among these 2 organisms MA1 growing on marine agar with 15% highest salt tolerant capacity in previously isolated *halomonas salifodinae* was growing 20% of salt concentration in media but in these research study they growing on 15 % salt concentration and NA2 growing on Nutrient agar in mesophilic salt concentration they growing same environmental condition like previously describe studies.

The PHB positive sp. showing gray black granules with covered a pink cytoplasm was observed through 100X oil immersion objectives of microscope. These granules were observed as same like previously describe studies.

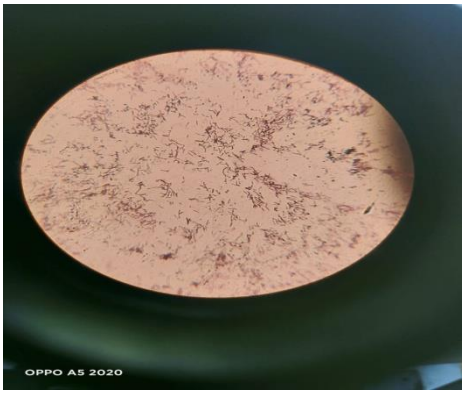

Fig: PHB staining

Biochemical characterization

The *MA1* and *NA2* isolated organisms are showing same results as previously describe biochemical test.

Table No. 1. : Biochemical characteristics of isolated organisms.

(*Shaima R. Banoon and Zahra M. Ali 2018*)

Characteristics	*Halomonas salifodinae(MA1)*	*Bacillus licheniformis(NA2)*
Morphology	Rod	Rod
Pigmentation	Yellow – creamy	Creamy
NaCl range	2 to 15%	2 to 10%
Temperature range	30 to 45°C	30 to 55°C
Temperature optimum	40°C	37°C

pH range	5 to 13	5 to 12
pH optimum	8	9
Oxidase test	Positive	Positive
Catalsase test	Positive	Positive
Hydrolysis of gelatin	Positive	Positive
Indol test	Negative	Negative
Methyl red test	Positive	Positive
VP test	Positive	Positive
Citrate test	Negative	Negative
Urease	Positive	Positive
Hydrolysis of Starch	Negative	Positive
Anaerobic conditions	Positive	Positive
Motility test	Positive	Positive
Gram's nature	Gram Negative	Gram positive

Phenotypic identification of the PHB positive isolates:

For a preliminary phenotypic identification, the two isolates were subjected to Gram staining and biochemical characterization. Gram staining revealed that MA1 isolates were Gram negative and NA2 were Gram Positive in nature; and based upon the results of the biochemical tests conducted, the two isolates were presumed to belong to *Halomonas and Bacillus species*. However, for further confirmation and species identification, the isolates were further examined by molecular techniques. In these organism *Halomonas salifodinae* sp. was previously isolated from *Mariout* salt lakes and *Bacillus licheniformis* sp. (*K.R. Shah 2014*) was isolated from soil, agricultural, sewage, industrial and other water effluents.

Molecular identification of PHB positive isolates:

For the identification of PHB producing bacteria, molecular techniques involving 16S rDNA amplification and analysis were used. The ribosomal operon, mainly 16S rRNA region is considered to be a stable and specific molecular marker for the identification of bacteria same procedure was followed by other studies for molecular identification of isolates.

Genomic DNA isolation from PHB producing bacteria:

Genomic DNA of the two PHB positive isolates (*MA1 and NA2*) was isolated by EDTA-phenol chloroform method with certain modifications. The quality and quantity of DNA samples was determined by electrophoresing the extracted DNA on 1% agarose gel. A single band of high molecular weight was obtained on agarose gel electrophoresis as show in fig A.

16S rRNA gene sequencing:

The 16S rRNA gene sequence is used to detect bacterial similarity as it is a highly conserved sequence. The genomic DNA of both the test isolates was subjected to PCR amplification by using universal primers and standard PCR conditions as described earlier. After resolving, sharp bands were observed at approximately 1500 bp. The PCR products after their cleaning-up were sequenced by out-sourcing, and the obtained sequences were subjected to computational analysis. Similar studies involving 16S rDNA analysis for identification of unknown bacterial isolates have been conducted by several earlier scientists (*Weisburg et al. 1991*).

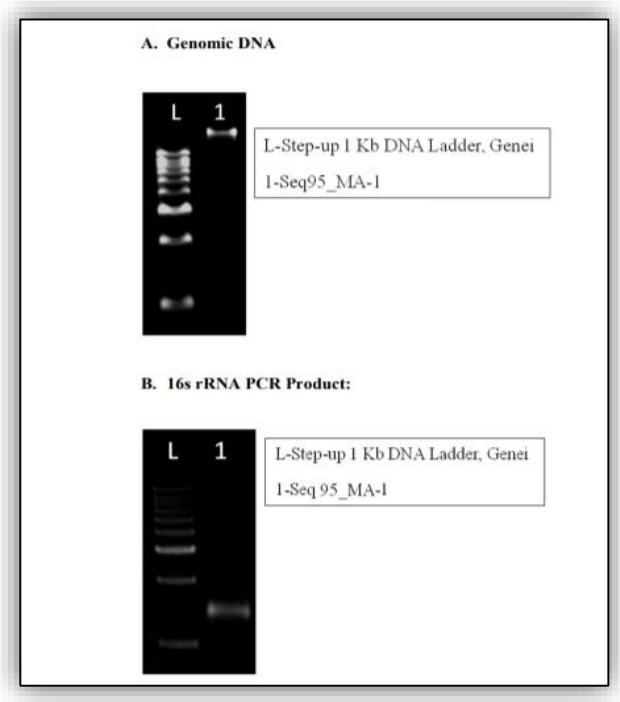

Fig: Isolation and PCR amplification of genomic DNA

Computational analysis of 16S rRNA gene sequence

The amplified 16S rDNA sequences were analyzed using BLASTN in NCBI database. BLAST looks for regions of local similarity among sequences, and is therefore an effective tool for understanding the functional and evolutionary relationships among sequences. BLAST analysis of the 16S rRNA gene sequences. The MA1 isolate showed DNA sequence similarity with *Halomonas salifodine (Fady Abd El-malek et. al 2020*). The NA2 isolates showing close DNA sequence with *Bacillus licheniformis* species. Further, the BLAST results were used for deriving phylogenetic relationships of both the isolates with homologous sequences showing98–99 percent homology with the two isolates.

Conclusion:

The main aim of this present study was to molecularly identify the PHB producing bacteria from marine water sample. Now a day's researchers are focusing on biopolymer producing microorganisms for developing biodegradable plastics. The 4 PHB synthesizing microbial strain was isolated in all four strains were the *NA2 and MA1* shows high amount of PHB synthesis. These two isolates were further biochemically and molecularly identified by National Chemical Laboratory Pune (CSIR), MS India. The higher PHB producing molecularly identified microorganisms will be selected further for optimization.

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