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EVALUATION OF PHB'S SYNTHESIZING MICROORGANISMS FROM MARINE WATER

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Abstract:

Plastic pollution is the buildup of plastic debris in the environment that negatively impacts people, wildlife, and their habitat. Plastic items are not biodegradable and they are still present in the environment. Bioplastics which are biodegradable plastics are emerging to solve the problem of plastic accumulation. Polyhydroxybutyrate or PHB is a bioplastic that serves as an alternative to synthetic plastics. PHB is a lipid reserve material that gets accumulated within the cell wall of micro organisms under stress conditions. In the current work, marine medium with a 15% high salt concentration was used to isolate halophiles. From the marine sample taken at Mumbai's Juhu beach, with using different selective medias such as Marine agar, Nutrient agar, Halophilic medium agar, Horikoshi agar and dunda's agar. During these no colony was observed on dunda's agar. 13 isolates were discovered among them from 11 isolates was showing PHB Positives. In this study isolated strain *MA1* Showing highest PHB production according to its spectrophotometric analysis of quantification. An isolated strain was morphological and biochemical characterized.

Keywords; Halophiles, Halophilic *Bacillus sp.*, Polyhydroxybutyrate, Marine medium, chloroform, spectrophotometer etc

Introduction:

One of the greatest problems that the world is facing today is that of environmental pollution, increasing with every passing year and causing grave and irreparable damage to the earth. Now a day's use of plastic and dumping it in environment it cause of environmental pollution. Humanity has quickly become addicted to plastic because of the low cost, stability, durability; good mechanical and thermal properties of plastic make it the best choice for widespread applications. However, the extensive use of materials made from plastics causes a worldwide problem because they are non-degradable.

Bioplastics, are alternative to the synthetic plastics, are the polymers produced by many microorganisms and are in turn are biodegradable to make biodegradable plastic is a better option for synthetic plastics. Bacterial polyhydroxyalkanoates (PHAs) are eco-friendly substitutes for petroleum-based

polymers. PHAs are biological macromolecules that are polyesteric and are reported outstanding because of their biodegradability and biocompatibility. The PHA types are polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH) and polyhydroxyoctanoate (PHO). Among these types PHB is the main biodegradable polymer. This PHB can be used as an effective thermoplastic, and has many characteristics similar to those of standard commercial synthetic plastics. Some bacterial species which naturally produce PHB are *Ralstonia eutrophes, Alcaligenes, Pseudomonas, Bacillus, Rhodococcus, Staphylococcus and Micrococcus etc*. PHB is eco friendly, biodegradable, biocompatible and is accumulated up to 90% of cell dry weight. Various bacteria from different environmental niches have been sourced for PHBs production and marine bacteria are rarely discovered for PHBs synthesis.

This research focuses on isolation and identification of PHB producing bacteria from marine water sample. The objective was to analyze the extracted PHB by different isolated organisms and quantified by spectrophotometric analysis. Biochemical and morphological tests are performed for the identification purpose. The marine environments provide an exciting resource for the discovery of new classes of PHB producers.

Materials and methodology

Marine water sample was collected from Mumbai juhu beach, India. Sample was collected in sterile plastic bottle, it transported to the laboratory aseptically and stored at 4 ^{0}C temperature in refrigerator for further use.

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. To make it's dilution to 10 $^{-3}$. For the isolation of organisms, 0.1 ml of each dilution was plated onto a nutrient rich medium by spread plate and streak plate method.

Testing of salt tolerance capacity

Prepare a dilution of enriched sample 10^{-1} to 10^{-3}. To take 10^{-3}diluted sample .it was streaking on Nutrient agar with different concentration of NaCl. Such as, 5%.10%, 15%and 20%.it was a method to cheek the salt tolerant capacity of bacteria in isolated marine water sample.

Selective Media's for isolation of Halophilic organisms from sample

There was choosing of 5 selective media's for isolation of halophiles such as; Nutrient Agar(NA), Marine Agar (MA), Dunda's Agar (DA), Halophilic Medium Agar (HMA) and Horikoshi Agar (HA), for the isolation of organisms. 0.1 ml of each 10^{-3} diluted sample was plated onto above a nutrient rich medium's.

Screening of PHB Producers

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. PHB positive organism showed blue black granules with pink colored cytoplasm.

Characterization of PHB producing isolates

PHB producing strains were identified and characterized by morphological and biochemical characterization according to the *Bergey's* Manual of Determinative Bacteriology.

Morphological characterization:

Morphological features were identified by growing the cultures on nutrient agar media and gram staining was performed.

Biochemical characterization:

Different Biochemical tests were carried out includes IMVIC tests, catalase test, urease test and starch hydrolysis.

Qualitative analysis of PHB production:

All the bacterial isolates were qualitatively tested for PHB production using Sudan Black B dye, for rapid screening of PHB producers. Nutrient agar medium was autoclaved and poured into Petri plates allowed for solidification, and inoculate each isolated bacterial culture. The plates were incubated at 42 ^{0}C temperature for 24-48 hrs. After incubation colonies was observed on plates. Than Ethanolic solution of (0.02%) of Sudan black was spread over the colonies and the plates were kept undisturbed for 30 minutes they were then washed with 98% ethanol to remove the excess stain from the colonies. The dark blue colored colonies were taken as positive for PHB production. [***Hartman1940].***

Quantification of PHB Production

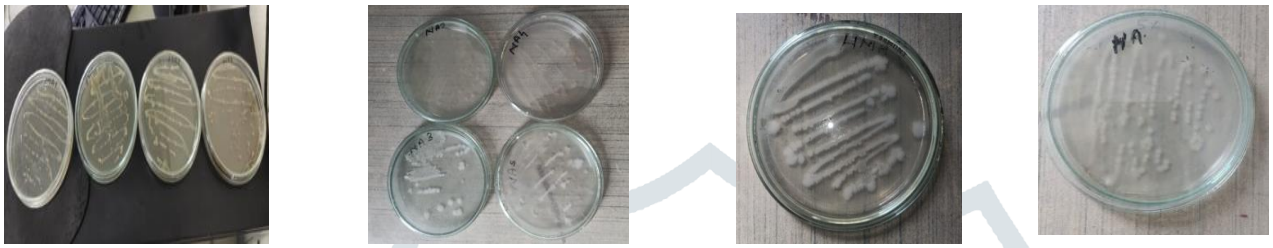
Take a broth containing PHB positive bacterial cells centrifuged at 10,000 rpm, at 4 ^{0}C temperature for 20 minutes. Take a pellet washed with equal amount of acetone and ethanol to remove the unwanted materials. Than pellet was resuspended in 5 ml of 4% sodium hypochlorite and incubated at room temperature for 30 minutes. The whole mixture again centrifuged at 10,000 rpm, at 4 ^{0}C temperature for 20 minutes. The supernatant discarded and take pellet it contains PHB polymer. Than pellet dissolved 10 ml of chloroform incubate it for 15-20 minutes it dissolve PHB in it. Then chloroform was filtered and to the felted, 10 ml of concentrated H2SO4 was added to the tube which was capped and heated for 20 min at 100 ^{0}C temperature in water bath. The addition of sulfuric acid converted the polymer into crotonic acid. The solution was cooled and absorbance read at 235 nm against a sulfuric acid blank (***John and ralpha 1961)***

Result and Discussion:

Isolation of microorganisms: Microorganisms were isolated from Marine water sample and independent colonies were obtained by serial dilution. A total of 12 bacterial colonies with different morphological features were selected using four different selective media's. In these 4 cololonies were isolated from marine agar the no giving to each colony such as; *MA1, MA2, MA3* and *MA4*. The 5 colonies were isolated from Nutrient agar the no. giving to each colony such as; *NA1, NA2, NA3, NA4* and *NA5*. The 2 colonies were isolated from

Horikoshi agar the no. giving to each colony such as; *HA1* and *HA2*. Only 1 colony were isolated from Halophilic medium agar the name giving to colony such as; HMA. No any colony was observed on Dunda's agar. The researcher *Nidhi patel et.al (2017)* they also isolated different Halophilic microorganisms from 4 different marine water sample. They were using HMA (Halophilic medium agar) for isolation of halophiles. They isolated among 21 different colonies but in this research study we isolated only 1 colony on HM Agar plates using only one marine water sample. These colonies were streaked on nutrient agar plates and preserved for further studies.

MA Plates **NA Plates** **HMA Plates** **HA Plates**



Salt tolerance capacity: It was checked using different concentration of NaCl such as; 5%, 10%, 15% and 20%. In this observation growth was not observed in 20% salt concentration. Salt tolerance capacity was not checked before to optimization, it was not observed in any study but it beneficial to isolate distinct salt tolerating halophilc colonies.

Fig: Salt Tolerance Capacity

PHB Screening: Among 13colonies, 11 colonies showed positive for Sudan Black B staining. Cell appears with pink color cytoplasm with blue black a granule was observed in light microscope. These 11 colonies named were *MA1, MA2, MA3, MA4, NA2, NA2, NA3, NA4, NA5, HA1,* and *HMA.* The PHB staining was previously used by researcher cell appears with pink color cytoplasm with blue black granule was observed on microscopic study. In these researches isolated strains showing same results. According to this study it concluded that *Christina thapa et.al* (2018) used method for PHB staining was appropriate.

Fig: PHB Staining

Morphological and Biochemical characteristics

Morphological features were observed for PHB producing strains. Strain *MA1, NA2, HA1* and *HMA* has bacillus in shape and strain *MA2, MA3, MA4, NA3, NA4, NA5* were cocci in shape. Strain *NA3* is pink in color, strain *MA2 & HA1* yellow in color and the remaining strains were cremish white in color. The different morphological characterized strain as like of *Christina Thapa .et.al. (2018)* they also were showing different colonies of different morphological characteristics like shape, color and its gram's nature. Strain *MA1 & NA2* is Gram positive and the remaining strains were Gram negative to gram staining. Morphological features were represented in Table 1.

Morphological Characteristics	MA1	MA2	MA3	MA4	NA2	NA3	NAA4	NA5	HA1	HMA
Shape	rod	cocci	cocci	cocci	rod	cocci	cocci	cocci	rod	rod
Color	cremish	yellow	white	white	cremish	Pink	white	white	yellow	white
Gram Staining	Gram + ve	Gram + ve	Gram - ve	Gram - ve	Gram + ve	Gram - ve	Gram - ve	Gram + ve	Gram + ve	Gram - ve

Table No. 1: Morphological characteristics of isolated strains.

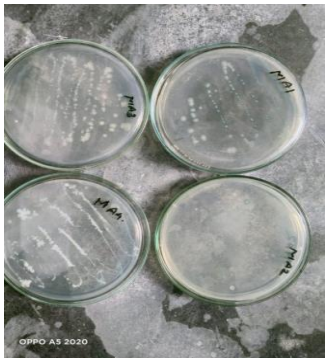
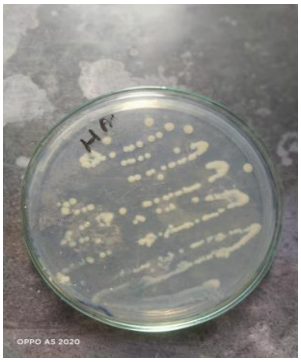
Different Biochemical tests have been performed for PHB producing strains. Strain *MA3*and *NA4* is positive to indole and the remaining are negative to indole test. All Strain are positive to MR test. Strain *MA2, MA4* and *NA3* are positive to VP test and remaining strains are negative to VP test. Only strains *MA2* was negative to citrate utilization test and the remaining strains are positive to citrate utilization test. Strain 2 and 4 are negative to starch hydrolysis test and the remaining strains are positive to starch hydrolysis test. Strain *MA2, MA4, NA5 a*nd *HMA* are negative to catalase test and other is positive. Strain *MA4* and *HMA* are negative to urease test and other are positive to these test. The different biochemical test used by *Sangita shakya and Rosy shakaya (2021)* used for isolated strains for identification. Same test and procedure was use in these research isolates for identification. The obtained result was compared with Begry's manual.

Biochemical Test	MA1	MA2	MA3	MA4	NA2	NA3	NA4	NA5	HA1	HMA
Indole Production	Negative	Negative	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative
MR	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
VP	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative
Citrate utilization	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Starch hydrolysis.	Positive	negative	Positive	Negative	Positive	Positive	Positive	Negative	Positive	Negative
Catalase	Positive	negative	Positive	Negative	Positive	Positive	Positive	Negative	Positive	Negative
Urease	Positive	Positive	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Negative

Table No. 2: Biochemical characterization of isolated strains.

Qualitatively Analysis: The dark blue colored colonies were confirmed qualitatively as positive for PHB production. The colonies showing blue colored zone was described by *Hartman1940 method.* It was used by *Lingayya Hiremath* (2015) in its research work. The same result was obtained in these research studies.

MA Plates **NA Plates** **HA plates** **HMA Plates**


Quantification of PHB Production: Higher concentration of PHB was observed in strain *MA1* by spectrophotometric analysis at 235 nm of isolates. The quantification of PHB production was used by previously described *john and ralpha* method (1961) used by *Lingayya Hiremath* (2015) in its research work. Extracted pellet of PHB was dissolved in chloroform than in adding concentrated sulfuric acid and boiled in water bath about 15-20 minutes after incubation it form brown color because of after treatment the dissolved PHB was converted into crotonic acid . The same color was observed in these researches. It gives different O.D. according to its crotonic acid concentration was obtained see in table No. 4. (Note: add extra sulfuric acid if reading is high)

Name of Strain	O.D (235 nm)
MA1	0.402
MA2	0.177
MA3	0.132
MA4	0.128
NA2	0.250
NA3	0.150
NA4	0.144
NA5	0.132
HA1	0.303
HMA	0.168

Table No. 3: Crotonic acid concentration.

Conclusion:

The main aim of this present study was to isolate the PHB producing bacteria from Marine water sample. Now a day's researchers are focusing on biopolymer producing microorganisms for developing biodegradable plastics. Among 13 isolates 11 isolates were PHB positive, in these 11 PHB positive strain's MA1 isolated strain showing highest PHB production. The PHB produced from this strain will further be characterized by analytical techniques like Infra Red spectra and Gas Chromatography analysis.

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