Making of Polythene by using marine and organic waste drive bacteria

[1]Santosh Kumar Vaish, [2]A K Gautam

[1]M. Tech Scholar, [2]Assistant Professor
[1,2]Dept of Civil Engineering,
Maharishi University of Information Technology, Lucknow, Uttar Pradesh

Abstract*: Bioplastics are biomass based biodegradable plastics which can be derived from corn starch, pea starch, vegetable fats and oils as well as microorganisms like bacteria, algae etc. They may be used for packaging purposes and catering items like bowls, pots, straws, cutlery etc., for making bottles for soft drinks, bags, trays etc. Plastic is one of the major pollutants at present time around the world, which is used for daily use like packaging materials, carry bags, manufacturing of different types of materials etc. So, to replace the use of synthetic plastic as well as to reduce the increasing environmental pollution an alternative must be developed. This need of synthetic plastic can be fulfilled by use of bioplastics. Polyhydroxyalkanoates are polymers produced by bacteria among which Polyhydroxybutyrate (PHB) is one major group. The property of PHB is similar to synthetic plastics. So, it can be used as a suitable alternative to the present day conventional practices for sustainability. Several bacterial species like *Actinobacillus*, *Azotobacter*, *Agrobacterium*, *Rhodobacter* and *Sphaerotilius* have been under focus for their ability of converting organic waste to bacterial PHA. For industrial production of PHB, some bacterial species like *Bacillus* spp., *Pseudomonas* spp., *Aeromonas* spp., *Cupriavidus* spp. have been extensively used for their potential to produce PHB. Since the production of bio-plastic is expensive many techniques have been adopted for large scale production. But, to obtain PHB in large amount the selection of proper strains of bacteria, capable of producing or accumulating PHB is necessary. Marine ecosystem is one of the largest ecosystems on Earth and still required to be explored. So in this study, comparison of the production of PHB (Bio- Plastic) in Marine and Soil bacteria has been done to find out which one has the potency to accumulate more PHB.

Keywords: Bioplastic, PHB, Synthetic plastic, Bacteria, Marine.

I. INTRODUCTION

Life – a beautiful word that holds many information within itself. It can be said that it is a system or object with many characters like self-sustaining and signalling mechanisms which differentiates them from other objects. Those others are called non-living objects or non- living systems. A system, in biology, can be defined as a group of organs which associate together to perform certain task. In chemistry, a system can be defined as any object of universe which is under observation or study. The systems always remain associated with its surroundings or environment. This association of systems and also interaction among them and their environment forms an ecosystem. The environment has a major role in the development of a system as well in its existence. These phenomena are regulated by various biotic as well as abiotic factors of an ecosystem. For existence of a system in an environment, it should always maintain a balance with its surroundings by its activity. Occurrence of any disturbance in the balance between the biological systems and their environment leading to a hectic situation in which it becomes uncomfortable for the living systems to live. Nature always tries to maintain this balance whereas anthropogenic activities may disbalance the same. Pollution is a condition in which contaminants are introduced in to the natural environments leading to adverse changes in the environment and human activity is the main cause for the same. Pollutants or contaminants are the components that cause pollution and thy may be foreign chemicals, substances (Fig. 1) or different forms of energy like heat, noise etc. Pollution may be point source or non-point source. The point source pollution is the type where pollution occurs in the same site where the pollutants are produced where as non-point source pollution is different from this type where the pollutants are carried to a different place from its origin via different transport media. Pollution may arise in different geographical locations leading to deformations in soil, water or air. Among different types, one newly discovered type of pollution is marine pollution, caused by various transport vehicles such as ship, ferry etc. and entry of various agricultural, industrial wastes into ocean water. Water from river and other water bodies flow and meet in the ocean. This carries various waste. Increased pollution over the surface of earth is creating critical problems in normal living conditions of human as well as other flora and fauna. The increase of temperature on Earth's surface is the result of ozone layer depletion and entrapment of greenhouse gases. In India, air quality data have been collected by NEERI (National Environmental Engineering Research Institute) from ten different cities of India such as Delhi, Kolkata, Mumbai, Chennai, Cochin, Kanpur, Nagpur, Hyderabad, Jaipur and Ahmedabad and from these data, Kolkata was found to be the most polluted city mostly with SO_2followed by Mumbai, Delhi, Ahmedabad, Kanpur, Hyderabad, Chennai, Nagpur and Jaipur. Jaipur was placed in the first position to be polluted with NOx. SPM (Suspended Particulate Matter) level was found to be highest in Delhi and Kolkata and lowest in Mumbai and Chennai. In Delhi, air pollution level was found to be highest among all other cities. According to a report, level of SO2 in atmosphere of Delhi has been recorded as 0.223ppm, whereas in Germany and USA 0.05 and 0.1 ppm are the permissible limits respectively. Methyl isocyanate leaked out from pesticide storage tanks in Bhopal, Madhya Pradesh, in 1984, killed over 3000 persons. The lead level of environment according to a guide of WHO is 2µg/m^3 (Verma and Agarwal, 2004). Many cities of India and various countries of world have crossed this level of lead. Excess growth of phytoplankton was first observed in the water bodies of Europe and North America.

Chemical wastes released from factories near Mirzapur, Uttar Pradesh has been reported to contain free chlorine which is the sole reason for the heavy mortality of fishes of Son River, Bihar.

II. SAMPLING AREA

1) Marine Bacteria: - A group of marine bacteria were sampled from sampling station that are Odisha cost in the Bay of Bengal. A couple of places from where the samples were taken were Chilka and Paradeep.

2) Organic Waste bacteria: - A group of different kinds of soil bacteria was deverived from the samples of organic waste that was obtained from a couple of garbage dumping locations in the South-East and North-East corners of Government Polytechnic Bargarh, Chitrakoot's campus.

III. MATERIALS AND METHODS

- **Marine Bacteria Isolation:-** A group f 10 distinct marine bacteria types were devised from sites of study such as the Odisha cost in the Bay of Bengal. A couple of places from where the samples were taken were Chilka and Paradeep. The specimen was taken in a flacon tine and was taken to the lab by preserving it on ice. These samples were then studies in laboratory with the help of serial dilution that takes place before spread plating in the nutrient agar plates to obtain a few separate colonies.
- **Isolation of bacteria from Organic-Wastes:** 20 different types of soil bacteria were isolated from organic-wastes samples collected from the two garbage dumping sites located at the North-West and South-East corners of Government Polytechnic Bargarh, Chitrakoot's campus. After collection of samples, serial dilution was performed followed by spread plating of the diluted samples in nutrient agar plates and incubated the bacterial culture plates at 37^{o} C for 24h.
- **Produced PHB Extraction in Potent Isolates:-** A couple of bacterial isolates, one of which was from the marine source Bacillus sp. (CS605) and another one from the waste Bacillus cereus (SE1) was taken for further research on the manufacturing of PHB that relies on the amount of brightness of granules of PHB that they produced. Their culture took place in Minimal David Media and was facilitated by dextrose acting as the source of carbon for about 3 full days at 37-degrees at about 150rpm in rotatory shaker. After a period of 3 days in incubation, PHB extraction takes place which is then followed by sodium-hypochlorite-chloroform method. A quantity of 5ml of the culture was rotates at a limit of 10,000g for about a couple of minutes and the outcome gets thrown away.
- **FTIR analysis of the extracted PHB:-** The PHB samples that were extracted were combined with KBr solution of 2%. Following this, the mixture was suppressed into translucent discs and this resulted in pellet and the process was initiated with a scan of 400 to up to 4000cm-1 (by Kansiz et al in 2000).
- **Portrayal of a Couple of Strong PHB Producers**

1) Gram Staining:- We take the full loop culture of a couple of isolates on 2 different glass slides and fix them with heat. These samples were stained with the help of primary crystals of violet stain and rested for about 30 seconds. More stain was washed off completely with the help of tap water and the acidic iodine was taken on the slide to rest for another 30 seconds. The slide gets washed again with some decolourizer or ethanol for a few more seconds and this process was followed by counter stain safranin to allow it to rest for another 45 seconds.

2) Scanning Electron Microscope analysis:- We take a Scanning Electron Micrograph of a couple of isolates for their morphology study and for comparing their size in a nutrient medium as well and minimal medium to ensure the production process of PHB. We take a 10ml sample of broth culture that is kept in a test flask. This culture gets centrifuged at a rate of 8000 grams at 4-degrees for about 5 minutes and following this, the cells were cleaned about 3 times with the help of 0.1M Buffer Saline Phosphate solution that contains a mix of KCL, KH2PO4, NaCl, Na2PO4 and the pH was 7.

3) Biochemical Test:- We perform the biochemical test to examine the utility of distinct sources of carbon offered in kits by a couple of isolates. About 50ul of this culture was taken in each of the wells of a biochemical kit and we then incubate these kids with distinct carbon sources besides the poring culture in a couple of isolates at about 37-degrees for a period of about a day.

4) Antibiotic Sensitivity Test:- For this test, containing a couple of isolates, about 100 ul of the culture was dabbed on what is known as Medium of Muller Hinton Agar (containing HiVeg of beef infusion at 2g per l, HiVeg Casein of acid hydrolysate at 17.5g per l, Starch at 1.5g per l, Agar at 17g per l, and pH at 7.3). Following this, about 5 distinct kinds of antibiotic plates were placed in a medium that was being incubated at about 37-degrees for a day

- **PHB Production Comparison of the Couple of Isolates of the Cell Population:-** A couple of strong producers of PHB was taken in a culture of low media that is enhanced with the dextrose source of carbon and after about 3 days of comparison of incubation of production of PHB in a couple of isolates of cell, population was done with the help of Flow Cytometry. These cells were then stranded in about 1 ml of saline phosphate buffered or PBS solution at the room temperature. Fluorescein Isothiocyanate or FITC at a proportion of 490 to 525 was suspended in a DMSO or Dimethyl Sulfoxide was mixed with the samples and the sample of the FITC dye, these cells were pellet with the final suspension of the 1 ml of PBS and this was stored in ice in a dark medium before its analysis. FITC that was 490 to 525 fluorescence was gauged with the help of a filter of bandpass (Kacmar et al in 2005).
- **PHB Production and Molecular Analysis of genes in a couple of strong isolates**

1) Template Preparation:- In order to prepare the template, the method of phenol-chloroform extraction was employed in which the foremost 300ul quantity of the bacteria grown overnight culture was obtained in a 1.5ml of eppendorf tube. Following this, we centrifuge the culture at about 600 rotations per minute for about 10 minutes and after this, we resuspend the pellet in a 567ul of TE buffer. Following this it was suspended at 30ul of the 10 percent SDS soln. and 3ul of the 20mg per ml of proteinase-K was mixed well, after which the incubation was done at 37-degrees for a day. After the incubation of 100ul of the 5M of NaCl was mixed to suspended solution and then mixed again well to centrifuge it for 6000 rotations per minute for 5. The supernatant solution was then added to a new tube. Following this, a single volume in the ratio 25 is to 24 is to 1 of Phenol and Chloroform ad

Isoamyl Alcohol was taken respectively and mixed well till there is DNA precipitate of stringy white in nature. Following this, the suspended solution was again taken for centrifugation at a rate of 10000 rotations per minute for about 5 minutes at room temperature. Following this, we take the supernatant waste and added to the 100ul of 70 percent ethanol. In the final step, the suspended solution was taken for centrifugation at about 10000 rotations for about 5 minutes and the pellet was then securely dried unto the evaporation gets completed of ethanol. Following this we add about 30ul of TE buffer. We check the purity of the DNA with the use of nanodrop and store it at -20-degrees until later use in a TE buffer.

2) Primer Usage Description:- For amplifying the phbA and phbB and phbC isolates genes, we use a trio of primers as per the report from Galehdari et al in 2009.

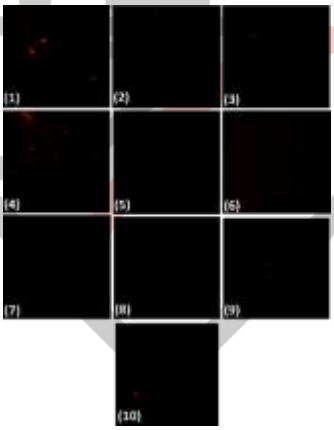
- **PCR Conditions Used:-**The reaction mixture of PCR comes with a 5ul of every primer, 5ul of sample DNA isolated out of the bacteria isolates, 5ul of PCR buffer, 5ul of MgCl2, 1.2ul of DNTP and 2ul of the polymerase of DNA. The conditions of the cycle contains a former denaturation of the 95-degrees for 5 minutes, after which there are 35 cycles of the denaturation at 95-degrees for about 2 minutes, an annealing process at 60-degrees for about 30 seconds, extension process of 72-degrees for about 2 minutes and a last extension of about 72-degrees for a time period of 10 minutes, after which a hold of 4-degrees was observed.

IV. RESULTS

Bacterial Strain Isolations from Organic and Marine Resources

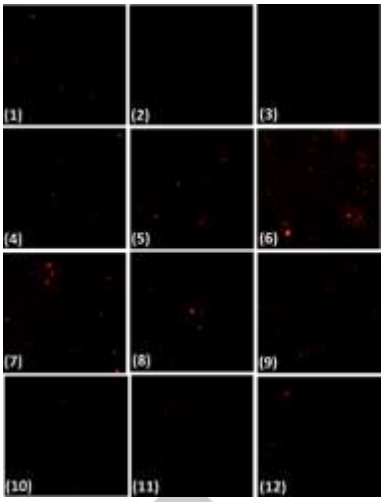
A sum of 32 isolates were taken from the marine resource (12 units) and organic waste (20 units) cultures sources on the agar Media of Nutrients.

PHB Production by Screening the Isolates:- We get the isolates from organic and marine resources by screen them for the production of PHB with the use of Nile Blue Staining and this was then viewed in fluorescence microscope where the colonies producing PHB gave out glorious orange colour.



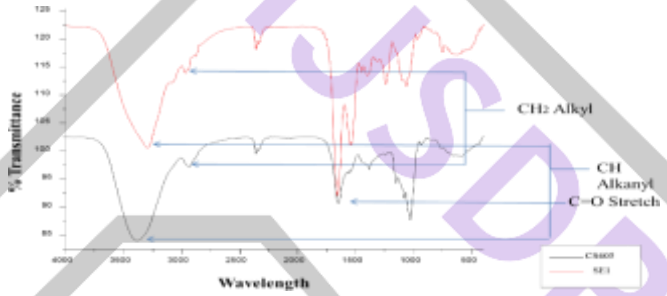
Organic Waste Isolates Collecting PHB

Many of the isolates got from organic wastes displayed the creation of PHB in the cells.



Extracted PHB characterization and FTIR reckoning

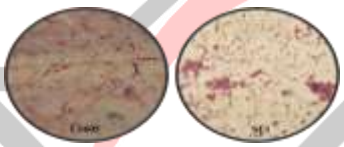
We perform FTIR analysis for determination of PHB extracts got from isolates that also gave the outcomes of a few peaks that showed the existence of a few function groups like CH2, C=O and CH etc. that are also a part of the structure of PHB



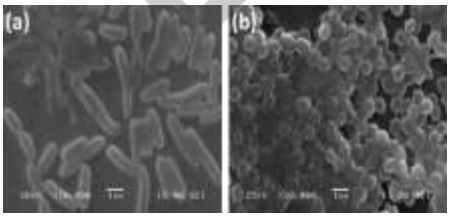
Description of Strong Isolates

Gram Staining

From the Gram Stain of a couple of strong isolates of CS605 was known to be the Gram positive Bacillus and SE1 was known to be the Gram positive Coccus



Scanning under Electron Microscope



Identifying Strong PHB Producers with Biochemical Tests

We perform a row of biochemical tests to know the unknown strong producers of PHB at CS605 and SE1

Distinct Sources of Carbon and Their Tests for Strong Isolate SE1

With the tests for employing distinct carbon sources that were done for knowing SE1 showed the below outcomes as in the Table below (according to the change in colors and patterns).

Test Conducted	SE1
Rhamnose	Negative
Cellubiose	Positive
Melezitose	Negative
α- Methyl-D-Mannoside	Negative
Xylitol	Negative
ONPG	Negative
Esculin	Positive
D-Arabinose	Negative
Citrate	Negative
Malonate	Negative
Sorbose	Negative
Control	Negative

Tests of Biochemical Species Except Use of carbon Source of SE1 identificatio

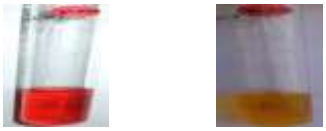
1) **Triple Iron Sugar Test**: With the help of this test, post a day of incubation, the media but that contained the dabbed culture was turning pink in color and the tilt of this culture turned yellow in color.

TSI before TSI after

2) **Citrate Use Test**: We see none of the changes in the culture of citrate media or SE1 after an incubation period of a day.

SCM before SCM after

3) **Use of Mannitol Test:** The hue of media after a day of incubation got changed to yellow from light red offering a positive test result.



Mannitol before Manitol after

4) **Test of Nitrate Reduction**: There were no special changes seen among the culture in the broth of nitrogen post and prior to a day of incubation.

Nitrate before Nitrate after

5) **Test of Gelatin Hydrolysis**: We tested the isolate that displayed a positive outcome during gelatin hydrolysis test. After its incubation, the media in culture was known to be the solvent that was in a semi-solid state prior to incunnation.


Gelatin before Gelatin after

6) **Production of Urease**: The outcome of production test for urease was known to be quite negative and there were no special changes in the media culture even after a day of incubation.


Urea before Urea after

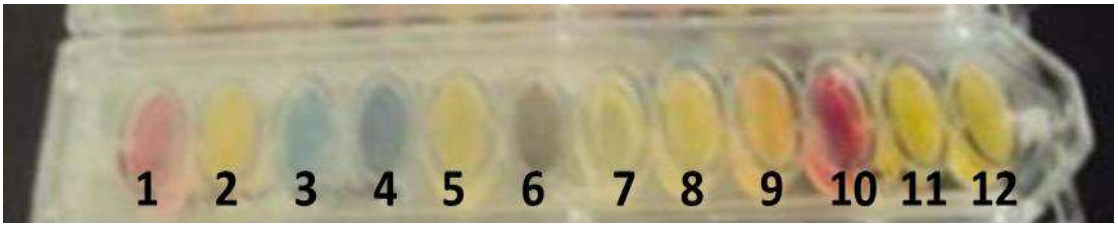
7) **Test for Oxidase Activity**: This is the test that displayed SE1 negative while CS605 in positive light for the oxidase activity.

CS605 **SE1**

Identifying CS605 with Biochemical Tests

We conduct a row of biochemical tests for knowing CS605 which displayed the results below

Tests conducted	Result
Methyl red	Negative
Voges proskeur	Positive
Citrate	Positive
Esculin	Positive
Urease	Positive
ONPG	Negative
Glucose	Positive
Sucrose	Positive
Rhanmnose	Positive
Malonate	Negative
Sorbose	Positive
Control	Positive Positive



According to the lots of biochemica test outcomes, the isolates were recognized as Enterococcus camalliae and Bacillis sp. for the SE1 and CS-605 respectively.

V. CONCLUSION

- Bioplastics are the best in this aspect. While organic and marine wastes contain a good amount of several nutrients and they also offer a lot of ecological strain conditions to the living people that are the last resource for the producers of PHB.
- In this aspect, the latest studies showed that the existence of a lot of producers of PHB in the couple of ecological system that can be employed for bioplatsic production at the industrial scale and even in a lab.
- The description of PHB by several analytic techniques came to the conclusion that producing purified PHB by the chosen isolates that can be researched again by several bending techniques to obtain a eco-friendly and inexpensive good.
- The strongest among all the isolates was known to be the Bacillus sp or CS605 and SE1 or Enterococcus camelliae. Bacillus spp are omnipresent in nature and have been known to get the ability of getting over the conditions of stress by several techniques.
- Though the Enterococcus spp. is known to produce PHB, low studies have now been conducted in this aspect.
- While the production of PHB and its ability was known to be the isolates of the marine source and organic wastes, the bacterial in this waste was known to be highly capable of the PHB production that may be as per the assertion that marine microbes are highly evolved due to de novo to outplay the conditions of stress.
- The ongoing search from several ecological conditions may offer some other appropriate isolates and the modifications of the genetic environment, for quick PHB production for commercial uses.

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