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Research Article

Biodegradable Plastic Poly Hydroxy Butyrate (PHB) Production by Bacterial Isolates from Marine Source and Plastic Waste

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Abstract

In the present study, PHB production has been observed more in plastic waste isdolate (52%) rather than from marine isolate. This property has been further increased by sodium alginate entrapment method to 61% in plastic isolate and 44% in marine isolate. The tensile properties and film formation of PHB were improved more with vinegar followed by PEG in comparision with the conventional plastic

Introduction

The history of plastic begins from 1862 by Alexander Parkes. The superior characters such as durability, strengths, shape and moldable oblige mankind incredibly depend on plastic for their daily life. Since these plastics have high molecular weight and tightly bonded together, these are not degradable, which makes their disposal difficult and inversely leads to negative impact on the environment [1].

Plastic a Major Environmental Pollutant

Accumulation of non degradable plastic bags in the environment is one of the major cases of pollutant in now a days. Only 1 to 2% of plastic bags gently recycled thousands of marine animals and more than 1 million birds die each year as a result of plastic pollution. Environment program estimates that there are 46,000 pieces of plastic litter floating in every square mile of ocean. Often mistakenly ingested by animals clogging their intestines, which results in death by starvation. Though plastics constitute only about 2.4% of the total municipal waste they are perceived as a major threat because of the long life and light weight [2].

In India, plastic waste accounts for only 0.6% of municipal solid waste. In urban areas of kerala, it is highly as 4-6% plastic accounts for approximately 10% of solid waste and contributes 80% of the waste accumulating on ocean surface [3].

Microbial plastics were polyesters that are produced by a range of microorganisms cultivated under various growth and nutrient conditions. These polymers usually lipids accumulated as storage lipids. Bioplastics were made from a compound polyhydroxyalkonate (PHB). PHB accumulated as energy reserve material in many microorganisms like *Bacillus, Nocardia, Rhizobium etc*. PHB is a highly crystalline thermoplastic polymer with a relative high melting temperature (170-180˚C). Wide production of PHB has far been limited due to high production costs [4].

Semi biodegradable plastics include starch-based plastics, protein based plastics etc. They can also be blended with conventional plastics like polyethylene (pt) mainly biodegraded partially. PHB are biodegradable plastics are similar to various synthetic plastics. Because they show complete degradation in nature. PHB's are ubiquitous in nature as they are found in Bacteria. PHB's and polyesters are accumulated by various bacteria under unbalanced conditions [5].

PHB has wide applications in different areas such as packaging material, long term dosage of drugs, medicines, insecticides, herbicides, fertilizers cosmetic world, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetics containers, shampoo bottles, cups etc. Studies are progressing for its relevance in medical field for bone replacements and plates, surgical pins, sutures, wound dressings, and blood vessel replacements [6].

Production of Bioplastic and its Future

The large production of bio plastic in industry is very much costly, so it has not been used extensively. Biodegradable plastics like polylactic acid, resins polyesters. 90% of total bioplastics demand, biodegradable plastics are environment friendly and can replace all plastic products available at this time production of bioplastics will definitely reduction in emission of CO_2 compared to traditional plastics. The cost of production of bioplastics is also too high. This is one of the major problems related to bioplastics development [7].

Strategies for Producton of Biodegradable Plastic

Biodegradation can be explained as a chemical process during which micro-organisms that present in the environment convert materials into natural substances such as water, carbon dioxide, and compost.

The term bio-based means the material is partly derived from biomass (plants). Synthetic plastics remain in the environment for long time as they are resistant to degradation [8]. Bioplastics are made from variety of sources like polysaccharides, lipids and also proteins. A few examples of protein used as substrates for bioplastic production are soy protein wheat gluten zein rice and egg albumin. Platicizer, which is a rupturing agent added with proteins to increase Plasticity. The accumulation of synthetic, petroleum derived plastics in the environment is a major cause of pollution. So the approach to produce plastic, which is an essential polymer used in our day to day life, using microbes (product of microorganisms) is a novel approach. It will reduce the environmental pollution as well as the use of petroleum to make plastic bags. So it can be said in one word that bio-plastic is eco-friendly [9].

Microbial Bioplastics

Microbes have been reported to be the potent producers of phb due to their high adaptability in various extreme environmental conditions. Out of these *Bacillus sps, Pseudomonas sps, Vibrio sps*. are found to be more efficient for PHB production due to their higher stability and reproducibility under environmental stress [10]. Some of the major groups of potential bioplastic producers have been discussed.

Bioplastics from Marine Environment

The advantages of using marine bacteria for the biosynthesis of PHA because of avoiding contamination with bacteria that lack salt water resistance. Biodegradable polymers are that anaerobic microbes completely degrade to water and CO_2 in various environments such as soil, sea, lake water and sewage and so it is disposable without harm to the environment.

Bacterial genera like *Beneckea* and *Vibrio* have been found to be first reported potent producers of PHA isolated from marine sediments [11].

Agro-Industrial Residues as Substrates in Bioplastic Production

Seven agro-industrial residues, *viz.*, wheat bran, potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder and corn flour were assessed for selecting the best substrate for PHB production. These were gelatinized, liquefied and saccharified as described by John *et al*. (2006) [12]. The conditions were: gelatinization at1000C for 15min, followed by liquefaction with alpha amylase (Novo Termamyl, 5000IU/ml) at 85˚C, pH 5 for 30min and then saccharification' Polyhydroxybutyrate production using agro-industrial residue as substrate with glucoamylase gave an yield of 2000IU/ml at 60˚C for 70min. The hydrolysate obtained was filtered through a muslin cloth and the clear hydrolysate containing reducing sugar was used as the sole carbon source for the PHB production. The reducing sugar in the hydrolysate was measured using dinitrosalicylic acid (DNS) method [13].

Industrial Production of Bio-Plastics

Even though the large-scale industrial production of bioplastics is costly, researcher are working to find out a better production by some potent PHB producing microorganisms using various types of substrates.

According to Kumar *et al*. (2004) [14], bacterial species present in activated sludge generated from a food processing industry are found to be potent for production of PHB. Bonartseva *et al*. (2004) [15] has shown that maximum PHB accumulates in Rhizobium lupine when grown in presence of glutamate and mannitol. Feed batch culture is one of the popular methods to obtain high cell density and large amount of desired product [16]. Wang and Lee (2007) have shown that nitrogen limited condition along with continuous feeding of sucrose increases the production of PHB.

Plasticizers and Additives for PHB

Plasticization and various parameters can indicate the features including polarity, hydrogen bonding, dielectric constant, and solubility parameters. Other important factor is solvation as plasticizers with solubility parameters close to those of the polymers requires less energy to fuse. The difficulty of processing plastic can be significantly influenced by the plasticizer type and concentration as well as other formulating additives, plasticizers can also be considered as processing additive. Biocompatable and biodegradable thermoplastics are with potential applications. PHB is one of the well known biodegradable plastic. PHB is a natural thermoplastic polyester and has many mechanical properties comparable to synthetically produced degradable polyesters [17].

Additives

The use of additives like, tributyrin, cause changes on the structure of PHB films decreasing their Tg and Tcc (cold crystallization) temperature. These additives are miscible with PHB, improved the mobility of the molecules in the amorphous phase. Besides acting as plasticizers these additives of small amount acts as accelerators for the enzymatic degradation of the polymer chains. The same effect was also observed by adding a biodegradable plasticizer di-n-butylpthalate (DBP) in PHB films.

Blending

One of the approaches being pursued to improve the performance of bio based materials in engineering applications is the blending of biopolymers with biodegradable synthetic polyesters. The goal of this approach is to develop biodegradable materials with acceptable material properties from blends comprising mostly biopolymers.

The main aim of this study was to identify the Bacillus genera in different soil samples and marine source and determine the amount of PHB production.

Objectives

1. Isolation and screening of bacterial species producing PHB from marine and plastic waste
2. Extraction of PHB from the fermentation medium.
3. Characterization of the two potent PHB producers
4. Determine the mechanical properties of PHB production
5. To assess the gel entrapment technique in PHB production.

Materials and Methods

Isolation of Marine Bacteria

Five different types of marine bacteria were isolated from study sites of Pulicat lake, Nellore District. The samples were then processed in the laboratory by serial dilution followed by spread plating in nutrient agar (Peptic digest of animal tissue 5g/l, Sodium Chloride 5g/l, Beef extract 1.5g/l, Yeast extract 1.5g/l, Agar 1.5%, pH-7.4±0.2) plates to get some isolated colonies. The spread plating was followed by incubation of the bacterial culture plates at 37˚C for 24h [21]. When the growth was proper, loop full cultures were taken from each single colony and streaked on culture plates containing nutrient agar medium to obtain pure culture of the isolates and this was followed by incubation of the plates at 37˚C for 24h. The pure cultures were preserved and maintained by sub-culturing the isolates at an interval of 1-2 weeks.

Isolation of Bacteria from Plastic-Wastes

Five different types of soil bacteria were isolated from organic-wastes samples collected from the two garbage dumping sites, SPMVV campus, Tirupati. 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˚C for 24h [18]. When the growth was observed in the plates, loop full cultures from different colonies were taken and streaked on culture plates containing nutrient agar medium as in the isolation of marine bacteria for obtaining pure culture of different isolates and incubated the plates at 37˚C for 24h. The pure cultures of different isolates of organic-wastes bacteria were preserved for future use in screening for production of bioplastic and maintained by sub-culturing the isolates at an interval of 1-2 weeks same as the marine isolates.

Screening of Different Bacterial Isolates of Marine and Plastic Wastes for Production of Bio-Plastic

To screen the cultivated marine and organic wastes bacterial isolates Nile Blue staining was performed. Bacterial isolates were cultured for 2-3 days at 37˚C in Minimal Davis Media (Dipotassium phosphate 7g/l, Monopotassium phosphate 2g/l, Sodium citrate 0.5g/l, Magnesium sulphate 0.1g/l, Ammonium sulphate 1g/l, pH-7.0 ± 0.2) supplemented with dextrose (10ml of 10% in 1l of Minimal Davis Media) as carbon source [19].

Nile Blue Staining

Loop full culture was taken on clean, sterile glass slides and heat fixed followed by staining with Nile blue stain. The samples were allowed to get stained for 20min at room temperature and then slides were washed with sterile water. Then the slides containing the samples were allowed to air dry for few minutes and observed under fluorescence microscope at wavelength 490nm. PHB granule producing bacterial isolates flourish bright yellowish-orange colour [20].

Extraction of Produced PHB in the Potent Isolates

Two bacterial isolates, one from marine source (CS605) and another from organic wastes (SE1) were selected for further study of production of PHB based on intensity of brightness of the PHB granules produced by them. They were cultured in Minimal Davis Media supplemented with dextrose as carbon source for 3 days at 37˚C at 150 rpm in a rotary shaker. After 3 days of incubation, extraction of PHB was performed following sodium hypochlorite-chloroform method [21]. Five ml of culture was centrifuged at 10,000rpm for 10 minutes and supernatant was discarded. The pellet was suspended in 2.5ml of 4% sodium hypochlorite for digestion and 2.5ml of hot chloroform was incubated at 37˚C for 1 hour. The suspension was centrifuged at 6000rpm for 10 minutes (The upper phase contains hypochlorite solution and the middle phase contains chloroform with cell debris). The bottom phase containing PHB with chloroform was collected and further was followed by extraction with hot chloroform and precipitated with ethanol and acetone (1:1). The precipitate was allowed to evaporate for dryness at 30˚C to obtain PHA crystals.

Determination of PHB

Alkaline digestion: The pellet was digested with 30% sodium hypo chlorite solution at 37˚C for 20mins. The spectrophotometric assay was done as described by Law slepecky (1979). The residue was collected by centrifugation at 8000Xg for 20 mins and performed a series of washing steps using water, acetone and finally ethanol. The polymer was dissolved in chloroform and kept for complete evaporation. Then 5ml of conc-H_2SO_4 was added and heated for 40mins at 100c in a water bath. The resultant crotonic acid was measured at 235nm against H_2SO_4 as a blank in photo spectrometry.

Characterization of the Potent PHB Producer

Gram staining and Scanning Electron Micrograph were performed to characterize their morphology. Different biochemical tests carried out to find out their sources for growth and development following the standard protocols [18].

Scanning Electron Microscope (SEM) Analysis

Scanning Electron Micrographs were taken of the two isolates for morphological study as well as for size comparison of the isolates grown in both minimal and nutrient medium to confirm the production of PHB. 10ml of broth culture was taken from the test flasks. Culture was centrifuged at 8,000g at 4˚C for 5 minutes and then the cells were washed three times with 0.1M Phosphate Buffer Saline (KCl 0.2g/l, KH_2PO_4 0.24g/l, NaCl 8g/l, Na_2PO_4 1.44g/l, pH-7.0). Then the cells were fixed by adding 2% Gluteraldehyde (prepared in 0.1M Phosphate Buffer Saline) followed by fixation of the cells by overnight incubation. Next day, cells were washed thrice with phosphate buffer saline followed by washing with 30%, 70% and 100% ethanol simultaneously. Then the fixed cells were incubated at 100% for 1hr. SEM stabs were prepared by applying adhesive tap and then applying the bacterial samples on the top [22].

Improving the Mechanical Properties by Adding Plasticizers

In order to increase the mechanical properties of PHB produced, plasticizers like vinegar and glacial acetic acid were added at the rate of 10% [17]. Different ratios were used for blending studies.

Preparation of the Blends

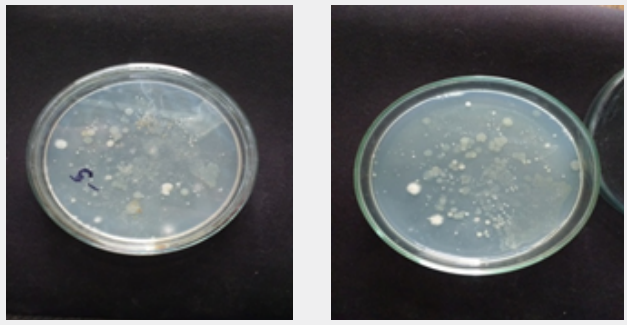
Blending of PHB/plasticizer blends was carried out in a Brabender Plastograph at temperature of 180°C, rotor speed 60rpm and mixing time 6 minutes. Before melting, PHB was dried at 50°C for 24h. Three types of blends (polymer/plasticizer) were prepared. The plasticizer was mixed with PHB before being loaded into the Brabender Plastograph. Once the melted products were obtained, were prepared sheets with dimensions (200 x 200 x 1) mm and films with thickness of maximum 100μm by compression-molding using a hydraulic press, at 175°C and 150atm pressure for 3min.

Entrapment Method

After 6hrs of incubation, sodium alginate solution and culture are added slowly with constant stirring and avoiding bubble formation. This makes the cells to get entrapped in the mattress of alginate slurry. The slurry is taken in the syringe and slowly dropped in the form of droplets in to $CaCl_2$ solution. The $CaCl_2$ gives stability to the sodium alginate gel in which cells are entrapped. The gel beads get hardened in the solution when it is left undisturbed for half an hour. Then these gel beads were washed twice with distilled water and these cell entrapped beads are preserved for further use [23].

Results

Total 18 isolates obtained. from marine 7 isolates were obtained.11 isolates from plastic wastes. Petri plates with black colour back ground represent marine isolates and blue colour background indicates isolates from plastic waste (Fig 1).



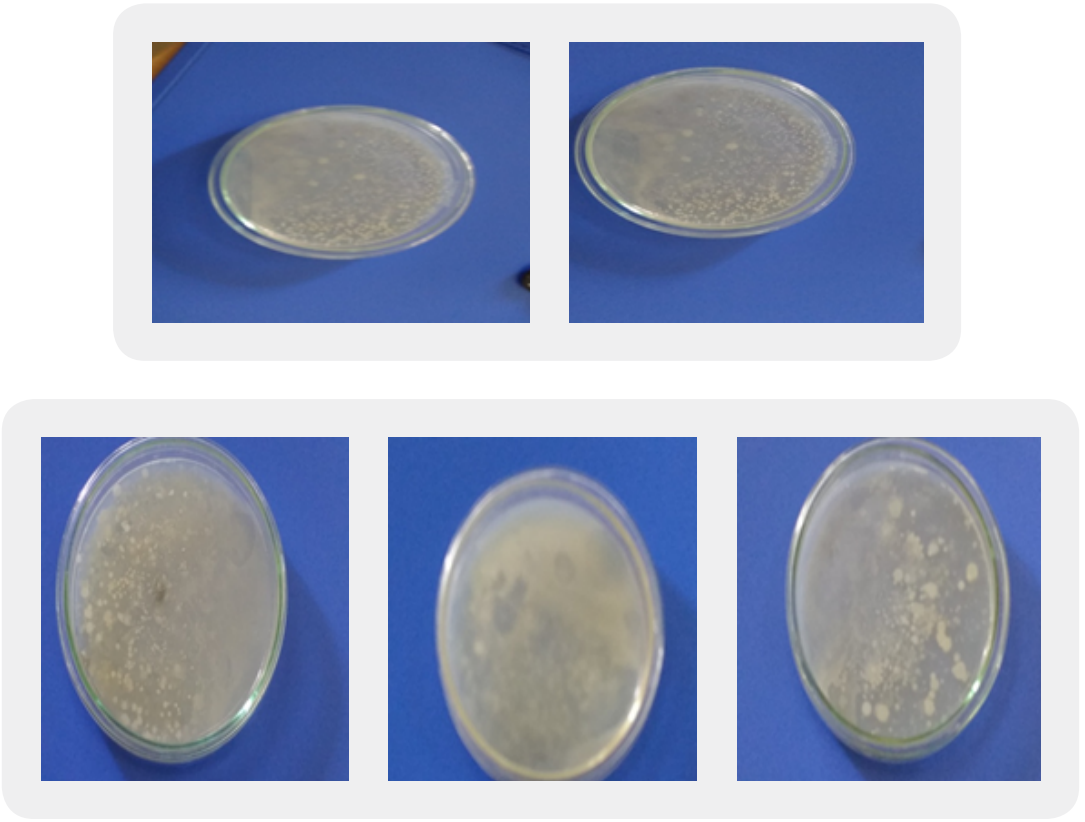


Figure 1: *Bacterial isolates from marine (Pulicat Lake) and plastic waste.*

Screening of the Isolates for Production of PHB

The isolates obtained from marine and plastic waste sources screened for PHB production using Nile blue staining method were observed under fluorescence microscope. PHB producing colonies fluoresced bright orange. (Fig 2)

Marine Isolates PHB Accumulation

Four of the isolates obtained from marine isolates shows the PHB production

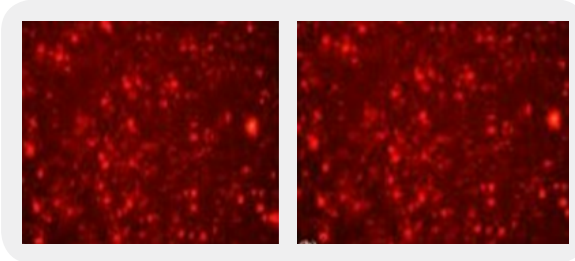


Figure 2: *Marine isolates with PHB granules appearing in bright orange colour*

Isolates of Organic Wastes Accumulating PHB

Five of the isolates of plastic waste was found to produce bright flourescence when observed under fluorescene microscope. (Fig 3)

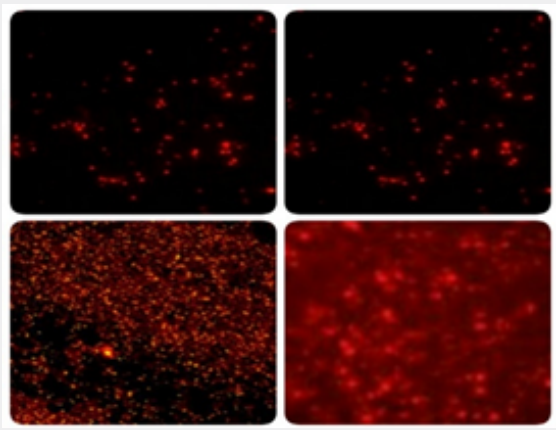


Figure 3: *PHB positive isolates from plastic waste.*

Characterization of the Potent Isolates

Gram Staining

From the gram staining method. The isolates were found to be gram positive *Bacillus* sps (Figure 4) and Table 1

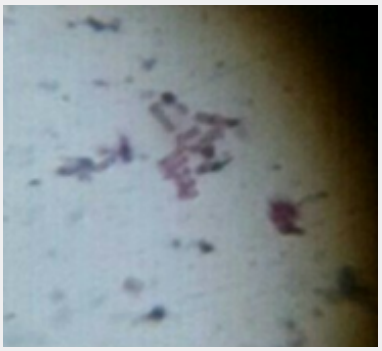


Figure 4: *Nile blue staining under light microscope*

Table 1: *Morphological Characterstics OF Bacillus SPS*

Colony Features	Result
Shape	Irregular
Size	Medium-large
Texture	Mucoid
Colour	Cream
Elevation	Flat
Density	Opaque
Margins	Irregular
Grams Reaction	
Grams nature	Positive
Shape	Rods
Size	Short
Arrangement	Chains/pairs

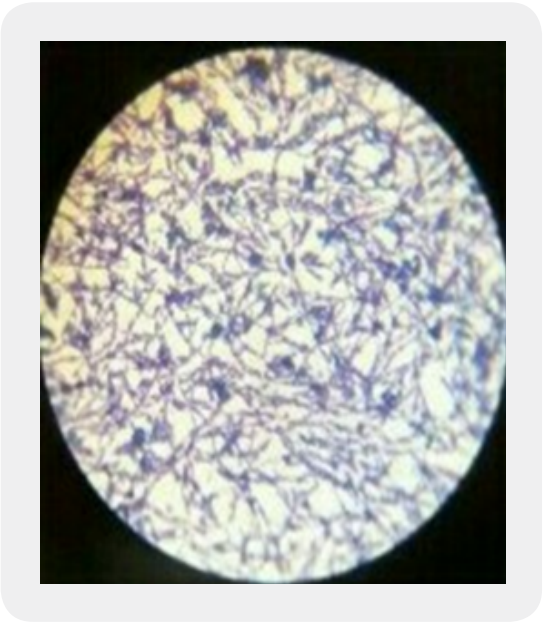


Figure 5: *Gram staining*

Scanning Electron Microscopy

From the staining, seven isolates out of 18 ,were gram positive rod shape when observed in scanning electron microscoy done at YAA Genomics, Chennai

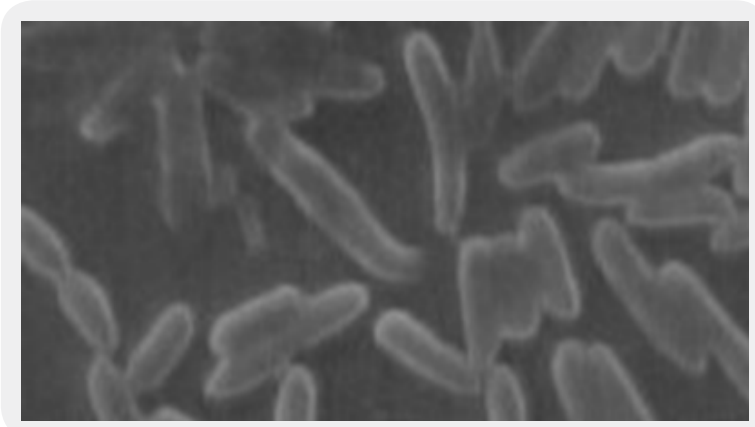


Figure 6: *Scanning electron micrograph of the major genera Bacillus sp.*

A series of biochemical tests conducted for identification showed the following results.

Table 2: *Biochemical tests.*

Tests Conducted	Results
Triple sugar iron test	+ve
Citrate utilization test	-ve
Mannitol utilization test	+ve
Nitrate reduction test	-ve
Gelatin hydrolysis test	+ve
Urease production	-ve
Oxidase activity test	+ve
Catalase test	+ve

Table 3: *Quantitative estimation of PHB*

Isolate	PHB produced in %	Amount of PHB expressed as crotonic acid
Marine isolate 1	8%	8mg/ml
2	17%	17mg/ml
3	26%	26mg/ml
4	35%	35mg/ml
Plastic isolate 1	44%	44mg/ml
Plastic isolate 2	53%	53mg/ml
3	32%	32mg/ml
4	48%	48mg/ml

5	8%	8mg/ml
6	21%	21mg/ml
7	15%	15mg/ml

Blending

The PHB was found to be brittle and breaks easily. Therefore blends with different polymers were prepared to improve their physical characterstics. In the present study, glacial acetic acid at the rate of 10%, polyethylene glycol, 2% GAA and vinegar at 5% concentration were added to the PHB. Further, tributryin is added to make the PHB film strong

PHB-GAA PHB –Vinegar PHB-PEG

Figure 7

Table 4: *Tensile properties of PHB in combination with plasticizers.*

S. No	Tensile strength (MPa)	Percentage of elongation (break)	Instability(˚C)
PHB	25	3-5	Above 200
PHB + glacial acetic acid	27	5-7	Stable upto 220
PHB+ Vinegar	30	4-7	235
PHB + PEG	28	8	240
Plastic	30-50MPa	10	250-300

Entrapment Method

Immobilized bacterial cells were observed by methylene blue mounting. Cells are immobilized in sodium alginate solution by entrapment method. Yield is calculated in terms of crotonic acid produced (Table 5).

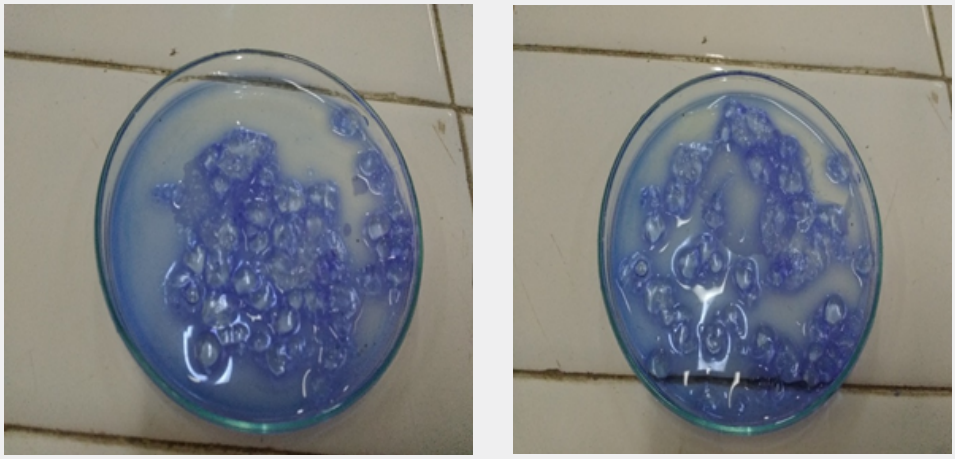


Figure 8

Table 5: *Effect of immobilization on PHB production.*

S.No	PHB Yield without entrapment	With Entrapment
Marine isolate	35	44
Plastic isolate	53	61
Plant based	46	52

Discussion

Plastic is one of the major pollutants now-a-days around the world. So, an alternative must be developed to replace this non bio-degradable pollutant, which is used by everyone in daily life. One should concentrate on the selection of proper strains of bacteria, capable of producing or accumulating PHB in large scale. Many reports are there for use of terrestrial bacteria capable of producing bioplastics [7], however, marine environments are the least explored compare to their terrestrial counterparts [24].

Marine ecosystem is one of the largest ecosystems on Earth and still required to be explored. So in this work, 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. *Bacillus* sp. from marine environment is found to be the most potent PHB producer in the current study which is in accordance to the previous results obtained by Lopez-Cortes *et al*. (2008) [25].

Precise techniques should be developed to extract PHB without any impurity so that the cost of production can be lowered. The feed stock required in fermentation is very much costly. So, at present time waste materials have been employed as biomass required for culture of microbes in PHB production.

Bioplastics as mentioned earlier are biodegradable plastics. So definitely they have specific half -life period. The degradation study of PHB also should be performed to find out the life span of product made from biodegradable plastics so that in future its quality and life span can be increased.

The production of PHB has been found more in bacteria isolated from plastic waste sources. The current result explored the plastic waste prominent in the terrestrial and non-marine environment

Plasticizers are important class of low molecular weight non volatile compounds that are widely used in polymer industries as additives. The primary role of substance to improve the flexibility and process ability of polymers by lowering the transition temperature, glass transition tempperature T_g. Plasticizer is a substance or material incorporated in a material to increase `its flexibility. These substances reduce the tension of deformation, hardness, density, viscosity, and electrostatic charge of a polymer at the sametime as increasing the polymer chain flexibility resistant to dielectric constant.

Cell immobilisation may be defined as confining the enzyme molecular to a distinct phase from the one is which the substrates as the products are present. There are different types of immobilisation of cells which include adsorption, covalent binding, entrapment and membrane. Confinement out of these entrapment is most widely used. In this enzyme are held to entrapped with in a bond formation between the enzyme molecule or matrix.

Summary

The current study revealed the presence of many PHB producers in both the environments studied which can be used for production of bioplastics in both laboratory as well as industrial scale. The characterization of PHB by various analytical techniques showed the production of pure PHB by the selected isolates which can be studied further by various blending techniques to get a more user friendly, bioproduct. The most potent among the isolates were identified to be *Bacillus* sp. *Bacillus* spp. are ubiquitous in nature and have been reported to possess the capability of overcoming the stress conditions by various mechanisms.. Hence, the continuous search from the various environmental conditions may provide some more suitable isolates for efficient PHB production for commercial use.

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. Plastic waste environment dwelling microbes are more potential to produce PHB and the selected isolates can be explored in future for commercial production of PHB.

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