Global Scientific JOURNALS

GSJ: Volume 6, Issue 7, July 2018, Online: ISSN 2320-9186
www.globalscientificjournal.com

PRODUCTION OF BIODEGRADABLE PLASTIC BY POLYHYDROXYBUTYRATE (PHB) ACCUMULATING BACTERIA USING LOW COST AGRICULTURAL WASTE MATERIAL

OMKAR J. AND ROHINI S.

Department of Microbiology, SS & LS Patkar College of Science, Goregaon (W), Mumbai 400062

Corresponding author e-mail :: omkarjathar678@gmail.com

ABSTRACT: Polyhydroxybutyrates (PHBs) are macromolecules synthesized by bacteria. They are inclusion bodies accumulated as reserve materials when the bacteria grow under different stress conditions. Because of their fast degradability under natural environmental conditions, PHBs are selected as alternatives for production of biodegradable plastics. The aim of this work was to isolate potential PHB producing bacteria and evaluate PHB production using agro residues as carbon sources. Among three bacterial strains isolated from different localities, only one PHB accumulating strain was selected and compared for its ability to accumulate PHB granules inside its cell. Isolate from Kalina Campus was identified as Bacillus spp and was found to be the best producer. The optimum pH, temperature, and incubation period for best PHB production by the isolate were 7, 37 °C, and 48 h respectively at 120 rpm. PHB production was best with glucose as carbon source and peptone as nitrogen source. The isolated *Bacillus subtilis* can be used for feasible production of PHB using agro-residues especially sugarcane bagasse which can reduce the production cost in addition to reducing the disposal problem of these substrates.

Keywords: *Bacillus subtilis,* Biodegradable, Polyhydroxybutyrates, Biodegradable

1. INTRODUCTION

Biopolymers are an alternative to petroleum-based polymers with a wide range of environmental advantages. Biodegradable materials under development include polylactides, polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymers and/or blends. Amongst these, PHAs are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a larger extent. PHAs are degraded to carbon dioxide and water in aerobic conditions and methane in anaerobic conditions by microbes 2 found in soil, water and other various natural habitats. Thus plastics produced from PHAs are truly biodegradable in both aerobic and anaerobic environments, unlike many of the "so-called" biodegradable plastics made synthetically. Plastic materials originated from petrochemicals cause serious environmental problems due to their non-degradable nature. Such synthetically produced polymers are generally inexpensive, but their persistence has a significant environmental impact. With the imminent fossil fuel crisis, the alarming rate of petroleum prices and environmental impact associated with the products, the search for alternatives is essential in reducing mankind's dependencies in non-renewable resources. Biodegradable plastics offer the best solution to protect the environment from hazards caused by conventional petroleum based plastics as they are 'eco-friendly in nature. There are many types of biodegradable plastics with different degrees of biodegradability. Among them polyhydroxybutyrate (PHBs) are the only 100% biodegradable ones. PHBs are macromolecules synthesized by bacteria and are inclusion bodies accumulated as reserve material when the bacteria grow under different stress conditions. They are polymers possessing properties similar to various synthetic thermoplastic like polypropylene. This makes them useful for extensive applications and future commercial mass production of biodegradable plastics that can replace plastic materials currently obtained from petroleum bases. However, a major problem for extensive production and commercialization of PHBs is their high production cost as compared with plastics derived from petrochemicals. Recently, much effort has been committed to reduce the production cost of PHB by using strategies such as; developing efficient bacterial strains, optimizing fermentation and recovery process. Most reports regarding the production of PHB suggested that, the major contributor to the overall PHB production cost was carbon substrate cost. As such, the selection of efficient carbon substrate is a key aspect, which verifies the total cost of the final product. The alternative approach is to choose renewable, economically feasible and most readily available carbon substrates for both microbial growths and efficient PHB production. Therefore, the objective of the present study was to isolate PHB producing bacteria and study its PHB production from agricultural waste materials.

2. MATERIALS AND METHODS

1. Sample Collection

Aseptic collection of samples was done from different localities such as i) Paddy field soil from sindhudurg region, ii) Soil from Kalina campus and iii) Garden soil from Vakola and properly cottoned to prevent contamination.

2. Isolation And Preservation Of Bacterial Strains

Serial dilution of the aseptically collected samples was carried out and streaked on Luria-Bertini Agar plates followed by incubation at 37˚C. Colonies were obtained and were then preserved on Luria-Bertini slants at 4˚C.

3. Screening For PHA Producing Bacteria By Sudan Black Staining

Colonies were then streaked on Luria-Bertini plates and then incubated again at 37˚C. The Luria-Bertini plates were then flooded with Sudan Black B stain. Observation was done for black colour colonies.

4. Identification Of PHA Producing Isolates

4.1 Gram staining

24 hr old cultures were smeared on a clean glass slide, air dried and heat fixed. The slides were placed on the staining glass rods. They were then covered with crystal violet for 1 min followed by washing with tap water. Then they were flooded with Gram's iodinezsolution for 1 min and then it was drained off. The smears were decolourized with alcohol for 30 seconds after which they were washed gently under tap water. The smears were counter stained with basic fuchsin for 1 min followed by washing under tap water. It was then air dried and observed under oil-immersion objective for gram nature.

4.2 Cultural morphology

Colony characteristics was studied by looking at the colony morphology and was represented in a tabular form.

4.3 Biochemical tests

Following biochemical tests were carried out-

a) Carbohydrate fermentation test

The saline suspension of the isolates were inoculated to the suspension tubes with St. Peptone water containing 1% Andrade's indicator with inverted durham's tube containing the following sugars: 1)1% glucose, 2)1% sucrose, 3)1% lactose, 4)1% maltose, 5)1% mannitol, 6)1% mannose and 7)1% xylose and incubated for 24 hrs at 37°C. The development of pink colour indicates a positive reaction.

b) IMViC test

ii) Indole test

The saline suspension of the isolates were inoculated to the suspension tubes containing St. Tryptone water and incubated at 37°C for 24 hrs. 1ml of Kovac's reagent was added along the side of the test tube. The development of a bright red colour ring at the inter-phase of reagent and broth constitutes a positive test.

ii) Methyl Red test

The saline suspension of the isolates were inoculated to the suspension tubes containing St. Glucose phosphate broth and incubated for 24 hrs at 37°C. 5 drops of methyl red was added to the tubes. The development of red colour was taken as positive for the test.

iii) Voges Proskauer test

The saline suspension of the isolates were inoculated to the suspension tubes containing St. Glucose phosphate broth and incubated for 24 hrs at 37°C. 1ml of Omeara's reagent was added to the tubes. The development of pink colour was taken as positive for the test.

iv) Citrate utilization test

Citrate utilization test was performed to find out the ability of the bacterial isolates to utilize or ferment citrate as the sole source of carbon. It was done on the St. Simmon's Citrate Agar slants and a change in the colour of the medium from green to blue was positive for the test.

c) Nitrate reduction test

The saline suspension of the isolates were inoculated to the suspension tubes containing St. Nitrate broth and incubated for 24 hrs at 37°C. 1ml of α-naphthylamine and 1ml of sulfanilic acid was added. The development of red colour was taken as positive for the test.

d) Urease test

The saline suspension of the isolates were inoculated to the suspension tubes containing St. Christensen's urea broth and incubated for 24-48 hrs at 37°C. The development of pink colour was taken as positive for the test.

e) Starch hydrolysis

The isolates were made a single streak on starch agar plate and incubated for 72-96 hrs at 37°C. Grams iodine solution was flooded on the surface of the plates for 30 seconds. The plates were examined for the starch hydrolysis around the line of growth of each isolates i.e., for the colour change of the medium. Clear zone surrounding the microbial colonies is a typical positive starch hydrolysis.

5. Production Of PHA By Selected Isolates

Culture was prepared by subculturing in M9 minimal broth. 1 ml culture was inoculated in the production medium. Incubation was done at 150 rpm for 48 hrs using rotary shaker.

6. Effect Of Carbon Sources On PHA Production.

The most promising isolate was incorporated in 4% of glucose in M9 at ph 7. Then the flask was incubated at 37˚C for 48 hrs at 150 rpm.

7. Effect Of Nitrogen Sources On PHA Production.

The most promising isolate was incorporated in varying concentrations of nitrogen in M9 at ph 7. Then the flask was incubated at 37˚C for 48 hrs at 150 rpm.

8. Extraction And Quantification Of PHA

10 ml culture was taken and centrifugation was carried out at 10,000 rpm for 15 mins. Supernatant was discarded. Pellet was then treated with 10ml sodium hypochlorite and incubated. Again centrifugation was done at 5000 rpm for 15 mins. Mixture was then washed with distilled water, acetone and methanol. Pellet was then transferred into a clean petri plate and weighed.

9. Measurement Of Dry Biomass

In a test tube 5 ml culture suspension was centrifuged at 10,000 rpm for 15 mins. Pellet was dried at 55˚C. Empty pertiplate was weighed followed by measurement of weight of plate containing dry biomass

10. Pre Treatment Of Agricultural Residues

Locally collected cane molasses was shredded into pieces and dried in an oven until it is completely dried and chopped into fine particles. It was hydrolyzed by zinc chloride method. Sugar content estimation was done by DNSA method.

11. Uv-Vis Analysis Of PHB Accumulation

Extracted PHB was dissolved in chloroform and scanned in the range of 235 nm against chloroform blank.

12. Preparation Of Bioplastic

The positive isolates were inoculated in M-9 minimal broth supplemented with best carbon source i.e. cane molasses and the best nitrogen source i.e. peptone at optimum concentrations and incubated under shaker conditions for 48 hrs. After 48 hrs, the bacterial cells containing the polymer were pelleted at 10,000 rpm for 10 min and the pellet washed with saline to remove the unwanted materials. The pellet was resuspended in equal volume of 4% sodium hypochlorite and

incubated at room temperature for 30 min. The mixture was again centrifuged and the supernatant discarded. The cell pellet containing PHB was again washed with acetone and ethanol. The polymer granules were then dissolved in chloroform. The chloroform was filtered and the filtrate was poured as a thin layer in a glass petri dish evaporated at 4°C, forming a film.

13. PHB Degradation Study

Bioplastic film obtained was dried in an oven at 60°C and later crushed into fine powder. The powder was incorporated in 100 ml flask containing M9 agar while it was in molten state, at pH 7. After stirring well, molten agar was poured into a clean petri plate and allowed to solidify. Plate containing PHB was inoculated with the same organism (*Bacillus subtilis*) and the plate was incubated at 37°C. The plate was observed on 7th day.

RESULTS

3.1 Isolation:

Isolation of the microorganisms was carried out by serially diluting the samples till 10^{-6}. The microorganisms were cultured on Luria-Bertini medium. As butyrate is a lipid molecule, logically, three mucoid colonies, one each from three soil samples were more focused during selection from large number of obtained colonies.

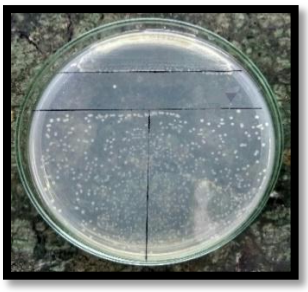


Figure 1: Paddy field count

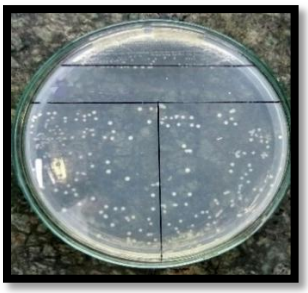


Figure 2: Kalina campus soil count

Figure 3: Garden soil count

3.2 Screening By Sudan Black B Staining:

Sudan Black B is a lipophilic dye that binds strongly to lipids hence used in the detection and confirmation of the presence of PHB granuels present inside the organism. The granuels are stained black which can be seen under a microscope. Here, the most strongly stained colony, that is, from Kalina Campus soil, was selected which is again stained with Sudan Black B and left

undisturbed for 30 mins followed by alcohol wash to remove excess stain and observed under a microscope.

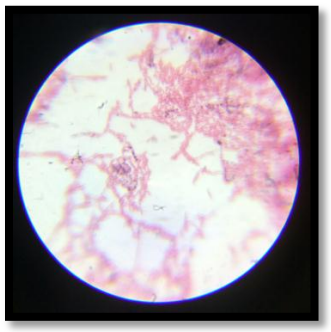


Figure 4(a): Field 1

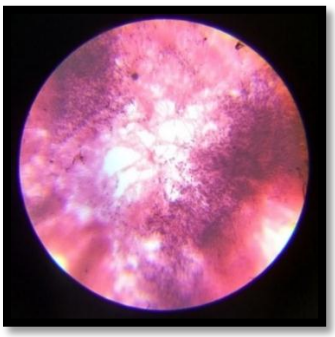


Figure 4(b): Field 2

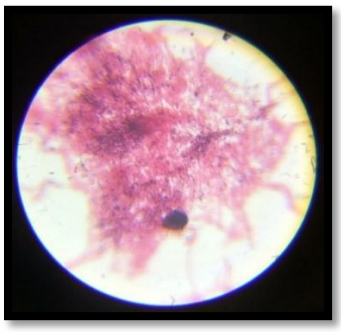


Figure 4(c): Field 3

3.3 Identification Of PHA Producing Isolates:

i) <u>Gram staining</u>

Gram staining was carried out and the isolate from Kalina Campus was found to be Gram positive rods which was then confirmed as *Bacillus*, using Bergey's Manual.

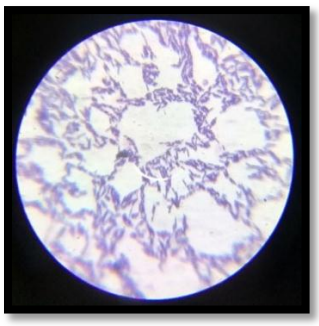


Figure 5: Gram Staining

ii) <u>Biochemical tests</u> Figure 6 represents the biochemical test results carried out with the purpose of identifying the bacterial species.

Figure 6: Biochemical test results

Table 1: Biochemical tests results of bacterial isolate

SR NO.	TESTS	RESULTS
A]	**CARBOHYDRATE FERMENTATION TESTS**	
1	Glucose	+
2	Sucrose	+
3	Lactose	+
4	Maltose	+
5	Mannitol	+
6	Mannose	+
7	Xylose	+
B]	**IMViC**	
1	Indole	–
2	Methyl Red test	–
3	Voges-Proskauer	+
4	Citrate	–
C]	**NITRATE REDUCTION TEST**	+
D]	**CATALASE TEST**	+
E]	**UREASE TEST**	–

KEY: + : Positive
- : Negative

From the above biochemical result, the promising organism was found as *Bacillus subtilis*.

Figure 7: Strach Hydrolysis Test

3.4 Effect Of Carbon Sources On PHB Production

Culture was inoculated in varying concentrations of glucose such as 2.5%, 5%, 10%, 15%, 20%, 25%, 30% and after successive incubation concentrations were measured colorimetrically.

Figure 8: Growth in different glucose concentrations

Table 2: Effect of different concentrations of glucose on PHB production by the selected bacterial isolate

ISOLATE	DIFFERENT CONCENTRATIONS OF GLUCOSE (%)	PHB YIELD (mg/100 ml)
Kalina soil	2.5	0.86
	5	1.62
	10	3.65
	15	5.18
	20	4.7
	25	3.5
	30	1.2

3.5 Effect Of Nitrogen Sources On PHB Production

Culture was inoculated in varying concentrations of nitrogen such as 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 and after successive incubation concentrations were measured colorimetrically.

Figure 9: Growth in different nitrogen concentrations

Table 3: Effect of different concentrations of nitrogen on PHB production by the selected bacterial isolate

ISOLATE	DIFFERENT CONCENTRATIONS OF NITROGEN (%)	PHB YIELD (mg/100 ml)
Kalina soil	0.1	0.94
	0.5	1.9
	1	2.8
	1.5	3.4
	2	3.9
	2.5	5.6
	3	4.7
	3.5	3.5
	4	3.2
	4.5	2.6
	5	1.4

3.6 Using Cane Molasses As A Carbon Source

Culture was inoculated in varying concentrations of Cane Molasses such as 2.5%, 5%, 10%, 15%, 20%, 25%, 30% and after successive incubation concentrations were measured colorimetrically.

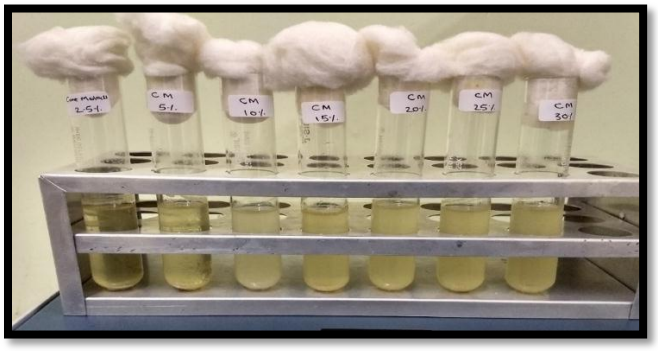


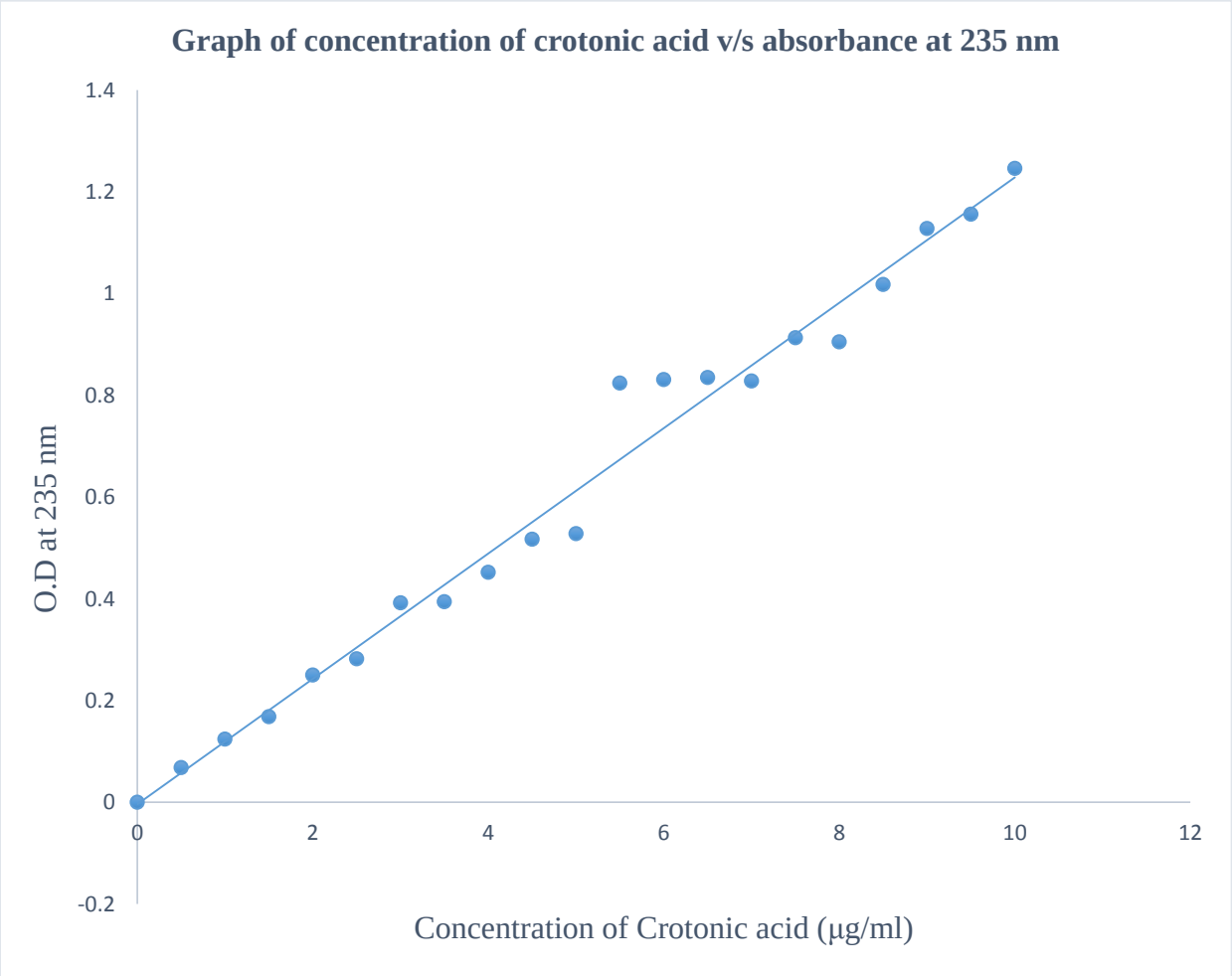
Figure 10: Growth in different Cane molasses concentration

Table 4: Effect of different concentrations of Cane molasses on PHB production by the selected bacterial isolate.

ISOLATE	DIFFERENT CONCENTRATIONS OF CANE MOLASSES (%)	PHB YIELD (mg/100 ml)
Kalina soil	2.5	2.8
	5	4.42
	10	7.86
	15	6.25
	20	3.63
	25	3.0
	30	2.3

3.7 Uv-Vis Analysis Of PHB Accumulation

Graph 1: Standard curve of concentration of Crotonic acid (mg/100 ml) v/s absorbance at 235 nm



Here, different concentrations of crotonic acid was measured against H_2SO4 blank using UV-Vis spectrophotometer at 235 nm. The absorbance of the sample was found to be at 0.60 nm and after plotting a graph of concentration of crotonic acid v/s absorbance at 235 nm, the concentration was found to be 4.9 µg/ml. As the sample was 1:2 diluted, the final concentration was found to be 9.8 µg/ml.

3.8 Preparation Of Bioplastic

Bioplastic was produced by the isolate from Kalina Campus using best carbon source (cane molasses) at 10% concentration and best nitrogen source (peptone) at 2.5% concentration and evaporating the filtrate at 4^{o}C.

Figure 11: Bioplastic

3.9 PHB Degradation Study

Bioplastic which was produced was degraded by the same organism by performing spot inoculation method. It might be due to the fact that due to starvation conditions the organism might be breaking down lipids to obtain energy.

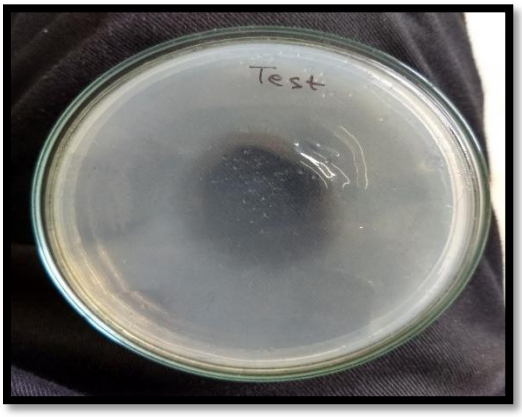


Figure 12: PHB degradation by *Bacillis subtilis*

4. CONCLUSION

The results of this study confirmed that cheaply available agro-residues can be used for the production of PHB serving triple purposes of reducing the cost of biodegradable plastics, reducing environmental pollution problems caused by conventional plastics and solving disposal problem of the agricultural wastes. The organism was able to grow well using cane molasses as a carbon source instead of glucose. It produced bioplastic in the presence of best carbon and nitrogen source at 4°C after pouring the filtrate into the plate.

Poly-β-hydroxybutyrate (PHB) is a natural, biodegradable polymer accumulated in the form of intracellular granules by a large variety of bacteria. In this project, PHB producing bacteria were isolated from different soil samples. The cultural parameters were optimized for the selected isolates. With a view to reduce the cost of PHB production, a cheaper substrate; molasses was evaluated for the production of PHB. Nowadays plastics and synthetic polymers are mainly produced using petrochemical materials that cannot be decomposed, thus resulting in environmental pollution. They are stored, burnt or recycled. During combustion, water and carbon dioxide are released into the atmosphere, i.e., an increase in the carbon dioxide concentration in the atmosphere occurs. Incineration is very difficult, dangerous and expensive and recycling is a long process and not very efficient. Some plastics still cannot be recycled or incinerated due to pigments, coatings and other additives added to the plastics when they are made. If plastics were made biodegradable, plastics would no longer accumulate as they do and recycling and incineration troubles would no longer be a problem.

5. DISCUSSION

Microorganisms play a significant role in biological decomposition of materials, including synthetic polymers in natural environments. High-density and low-density polyethylenes are the most commonly used synthetic plastics and they are slow in degradability in natural environments, causing serious environmental problems. In this regard, there is a growing interest in non-degradable synthetic polymer biodegradation using effective microorganisms.

Plastics have become an important part of modern life and are used in different sectors of operations like packaging, building materials, consumer products and many more. Each year, about 100 million tonnes of plastics are produced worldwide. Most of the plastics and synthetic polymers are produced from petrochemicals. Because of their persistence in the environment, several communities are more sensitive to the impact of discarded plastics on the environment including deleterious effects on wild life and on the aesthetic qualities of cities and forests. Plastic bags or sheets do not allow water and air to percolate into earth causing reduction in fertility status of soil, depletion of underground water sources and damage to animal life. In seas too, plastic wastes choke and entangle the marine mammals. In cities, they choke drains leading to submergence of roads especially during rainy season. The increased cost of solid waste disposal as well as potential hazards from incineration of wastes such as dioxin emission from PVC makes synthetic plastic waste management a problem (Ojumun et al., 2004). Consequently

hence, for the past two decades, there have been a growing public and scientific interest in the development and use of biodegradable polymers as an ecologically useful alternative to plastics. Biodegradable plastics are made from renewable resources and do not lead 82 to depletion of finite resources. Polyhydroxyalkanoates (PHA) synthesized by at least 75 different genera of microorganisms are attracted as biodegradable plastics. They are accumulated intracellularly, as high as 90 per cent of cell dry weight under conditions of nutrient stress and act as a source of carbon and energy (Madison and Huisman, 1999). Hence, in this project, PHB accumulating bacteria were isolated from diverse sources to select the efficient strains. The process parameters for maximum PHB production were also optimized.

In the present study potential PHB accumulating bacteria were isolated from diverse sources and potential strains were selected for further studies. Most of the potential isolates were Bacilli. Bacillus sp are reported to be ideal PHB producers. The optimum growth and the maximum PHB accumulation by isolate happened at 48 hrs. This shows biomass and PHB production were concomitant with growth conditions and PHB production of a particular strain is related to its biomass. As biomass increases the bacteria starts accumulating PHB to the maximum level and the accumulated PHB decreases after the peak biomass production. This might be due to nutrient depletion, which forces the bacteria to use the accumulated PHB as energy source. The highest yield of PHB was obtained with glucose (15%) after 48 h incubation. Glucose is an easily assimilable carbon source that encourages bacteria to produce more PHB. Pre-treated sugarcane bagasse (15%) was the best cheap carbon source followed by corn cob. Similar results were reported by Yu et al. who obtained 54% PHB using bagasse hydrolytes from *Cupriavidus necator*. Paramjeet et al. obtained 60% PHB from sugarcane bagasse by *Pseudomonas aeruginosa.*

The optimum temperature for growth and accumulation of PHB by isolate was 37°C. The PHB and biomass yields increased till 37°C and sharply declined at temperature extremes. Bellard et al. also reported maximum cell density and PHB accumulation at 37°C after 48 h. The alteration in the PHB content by temperature variance can be due to the fact that extreme temperatures slow down the metabolic activity (enzyme activity) of microorganisms that ultimately reduces their ability to produce PHB. The maximum PHB production percentage per dry cell weight was achieved with peptone as a nitrogen source. UV–Vis scanning of the extracted polymers showed peaks between 235 nm readings. This peak range indicates the occurrence of PHB. The plastic nature and biodegradability of the extracted polymer was confirmed by preparing sample plastic film and the clear zone formed by soil born bacteria. PHBs are degraded by the action of microbial enzyme, PHB depolymerase, into water-soluble forms.

REFERENCES

[1] Anderson and Dawes (1990) "Evaluation of soil Gram-negative bacteria yielding polyhydroxyalkanoic acids from carbohydrates and propionic acid" *Appl Microbiol Biotechnol. vol 3*: pg.216-229

[2] Bellard, Gurieff N., Lant P. (1995) "Isolation and screening of Polyhydroxybutyrates producing bacteria from pulp, paper, and cardboard industry wastes*". Int J Biomat*. vol 4: pg.302-310

[3] Doi and Lee (1996) "Plastics from bacteria and for bacteria" *Handbook of lipid and hydrocarbon microbiology*, vol : pg.196-205

[4] Jackson and Hrabak, (2009), "Polyhydroxybutyrate production using agro-industrial residue as substrate" *Arch Biol Technol*. vol 3: pg.567-574

[5] Thakur Pooja (2009)*"*Screening of plastic degrading bacteria from dumped soil area*" Acad. Indus. Res*. vol 2: pg. 18-22

[6] Yilmaz , Waqas N., Chaudhry N., Jamil L., Ali M., Hashim A., Shahida H. (2005), "Isolation and identification of PHB producing bacteria" vol 8: pg.395-406

[7] Yang J, Yang Y ,Wu WM ,Zhao J ,Jiang L (2014) "Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating waxworms*". Environ Sci Technol*; vol 48: pg.136–144.

[8] Zhang, Yu PH., Chua H., Huang AL., Lo W., Chen G. (1994) "PHA production by recombinant bacteria*" Bioresour Technol*. vol 2:4 pg.82-495