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RESEARCH ARTICLE

ISOLATION OF SEAWEED ASSOCIATED BACTERIA'S AND THEIR PRODUCTION OF BIOPOLYMER BY SOLID-STATE FERMENTATION

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Abstract

Plastic material is any of a wide range of synthetic or semi-synthetic organic solids that are moldable. Plastics are typically organic polymers of high molecular mass, but they often contain other substances. They are usually synthetic, most commonly derived from petrochemicals, but many are partially natural. Environmental pollution by the disposal of non-degradable conventional plastics is a fast-growing problem worldwide, especially in India. Nowadays, plastics are replacing by other constructional materials like glass, wood, metal in numerous applications. In this study, there has been considerable interest in the development and production of biodegradable polymer tosolve the current problem of pollution caused by the continuous use of synthetic polymer from seaweed associated Bactria. . Based on the dry weight of total biopolymer content, the strain SBT 09 showed maximum accumulation and selected for optimizing at different pH, temperature, salinity, carbon, and nitrogen source and the peak time of PHB accumulation. Based on the biochemical tests, the strain SBT 09 was found.The optimum pH, temperature, and salinity were found to be 7, 300C, and 5%. The peak time of PHB accumulation was found to be 36hrs. The best carbon and nitrogen source was found to be sucrose and (NH4)2 HPO4. The selected strain was mass cultured using the optimized media and PHB was extracted by solvent extraction.

Keywords: Marine Bacteria, PHB, Biosynthesis, Biopolymer

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Introduction

Plastic material is any of a wide range of synthetic or semi-synthetic organic solids that are moldable. Plastics are typically organic polymers of

high molecular mass, but they often contain other substances. They are usually synthetic, most commonly derived from petrochemicals, but many are partially natural. Environmental pollution by the disposal of non-degradable conventional plastics is a fast-growing problem worldwide especially in India. Nowadays, plastics are replacing by other constructional materials like glass, wood, metal in numerous applications (Poirier *et al*., 1995). According to the Comptroller and Auditor- General's (CAG) report, there are over 1.5 Million Tons of plastic wastes are generated in India annually and it has been estimated that 56 % of all plastics waste is used as packaging, and mostly originates from households. These synthetic polymers are a major pollutant in the environment because they are highly stable and thus remain persistent in the environment for decades. In worldwide there is only 7 % of total plastic waste is currently being recycled and the remaining un-recycled plastic accumulates within landfills sites and oceans (Lemos*et al*., 2006).

In general, plastic production also involves the use of potentially harmful chemicals, added as stabilizers or colorants and many of these chemicals have not undergone environmental risk assessment and their impact on human health and the environment is currently doubtful (Reddy *et al*., 2003). The increased usage of plastics in daily life and its pollution problem in the environment has motivated researchers and scientists to bring alternative eco-friendly biodegradable polymers with plastic-like properties as an alternative with greater compatibility to the environment (Braunegg*et al*., 2004; Akiyama *et al*., 2003). Plastics are utilized in almost every manufacturing industry ranging from automobiles to medicine. Plastics are very much advantageous because as synthetic polymers, their structure can be chemically manipulated to have a wide range of strengths and shapes. They have molecular weights ranging from 50,000 to 1,000,000 Da (Madison and Huisman, 1999).

Synthetic polyethylene, polyvinyl chloride, and polystyrene are largely used in the manufacture of plastics. Plastics can be easily molded into almost any desired shape including fibers and thin films. They have high chemical resistance and are more or less elastic, hence popular in many durable, disposal goods and as packaging materials.

Plastics degrade very slowly in nature. Because of their intermolecular bonds, some plastics may persist in the environment for thousands of years. Because of the low density they also tend to float in water (Joblin., *et al*.,1995)Human activities are responsible for a major decline of the world's biological diversity, and the problem is so critical that combined human impacts could have accelerated present extinction rates to 1000–10,000 times the natural rate (Lovejoy, 1997). Pollution is a condition in which contaminants are introduced into the natural environments leading to adverse changes in the environment and human activity is the main cause for the same. Accumulation of non-degradable plastic bags in the environment is one of the major causes of pollution nowadays. A statement, given by Supreme Court, says that the plastic bags threat is more serious than the atom bomb. Only 1 to 2% of plastic bags in the USA end up getting recycled. Approximately 380 billion plastic bags are used in the United States every year that is more than 1,200 bags per US resident, per year. Approximately 100 billion of the 380 billion are plastic shopping bags. Thousands of marine animals and more than 1 million birds die each year as a result of plastic pollution. Thus the present study is aimed to isolate and identify the potential PHB producing bacterial strains from the coastal dune rhizosphere soil, to optimize and synthesize biopolymer to solve the above-mentioned problems.

Materials and method

Collection of samples:

The seaweed-associated water sample was collected from Parangipettai, Cuddalore, Tamilnadu, India.). The samples were collected using a sterile spatula and aseptically transferred into sterile polythene bags. To avoid further contamination they were stored in an icebox (4^0C) and bought into the laboratory, PG and Research Department of Biotechnology,Sri Vinayaga College of Arts and Science College,Ulundurpet, Tamilnadu, India.

Isolation of bioplastic producing bacteria

The sample was suspended in 90ml of sterile distilled water blank and shaken vigorously for 2 mins. It was serially diluted up to 10^{-6}. The 0.1ml of the sample was taken from the dilution 10^{-4}, 10^{-5}, and 10^{-6} and spread in sterile E-2 mineral medium (Norris and Ribbon., 1971). The plates were incubated at 28±2 for 48 hr. in practice; nitrogen limitation was used to isolate bacteria rich in polycultures thus obtained were inoculated into sterile E-2 mineral agar slants for further use.

Morphological identification of isolated organisms

The PHB produced 30gum like colonies were observed in the E2 media plate. These colonies were morphologically identified. Different strains were obtained from seaweed-associated water samples, the morphologically identified 30 strains were streaked with E2 mineral medium. The morphologically identified 30 strain was stored in an Eppendorf tube. These strains are sub-cultured in an E2 mineral medium.

Screening of phb producing strain using sudan black staining:

Bacteria accumulating PHBs can also be easily identified on solid medium, as they appear as more turbid gummy-like colonies than the cells not producing PHBs (Sclegel, 1970; Ostle and Holt, 1982 and Kitamura and Doi, 1994). However, for the definite visualization of PHB producers, staining methodologies are useful (Kim *et al.*, 1996). The poly-β-hydroxybutyrate stain is used to stain granules are storage components of the cells indicating the cell's ability to take advantage of an opportunity of "free" easily absorbable and available low molecular weight carbonaceous compounds present in the environment. In the present study, turbid gummy-like colonies were isolated and Sudan Black-B staining was done for confirmation.

Identification of PHB-producing strain.

Using gram staining and biochemical characterization, identification of the PHB producing bacterial forms were done following the scheme of Cappuccino and Sherman (2002), and the result was also cross-checked with Bergey's manual of determinative bacteriology

Optimization of media for PHB Production:

Optimize the growth conditions for PHB production the selected strains were inoculated in E2 mineral medium with different pH (3.0, 5.0, 7.0, 9.0 and 11.0), saline concentration (0.5%,1%,1.5%,2.0% and 2.5%) different carbon source at 0.3% (Sucrose, Lactose, Maltose, Glucose, Cellulose) and nitrogen source at 0.3% (NH4(HPO4), KNO3, NH4cl, Tryptone and Peptone). The determination of peak time of PHB accumulation was determined at different time intervals.

Mass scale culture:

For mass culture, a total volume of 1000ml of the above optimized E-2 mineral medium was prepared and sterilized**.** The selected strain was inoculated into the medium and incubated with gentle shaking for 36hrs.

Extraction of phb:

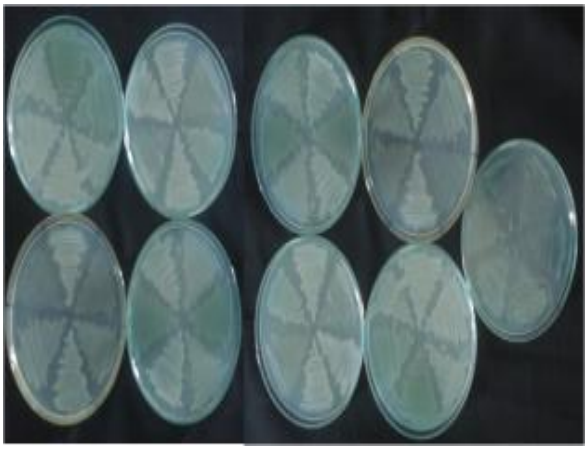
(Anwar *et al.*, 2005 and Bonartseva and Myshkina, 1985)

Result

The Biopolymers from bacteria will helps to produce biodegradable products in large quantities at a very low cost. Microbes belonging to more than 90 genera-including aerobes, anaerobes, photosynthetic bacteria, archaebacteria, and lower eukaryotes can accumulate and catabolize these polyesters (Lorenzo and Silvestre 1999). So, therefore focusing research in these areas will help to completely avoid the usage of non-biodegradable products in the future. In this present study, an 11seaweed associated water samples, were using E-2 Mineral medium (Plate 1), among the 12 samples the maximum colonies were 1.27 X 10^5 CFU/g (Fig. 1). For this, morphologically 58 different strains, were isolated from 12 different samples, based on the turbid gummy-like appearance 32 strains were selected for Sudan black staining and Gram staining. Among the 48 strains, 32 strains

showed gummy and sticky surface colonies and they were named SCBT 5101 to SCBT 5132**.**

Plate 1



Gram staining results were showed that most of the bacteria belong to gram negative (Table 1). The dominance of Gram-negative bacteria in the aquatic environment is due to their cell structure. Marine environments are nutritionally dilute when compared with the terrestrial environment. Under such conditions, the outer membrane, especially the lipopolysaccharide (LPS) of Gram-negative bacteria helps to absorb nutrients. In general, PHB is produced on an industrial scale using Gram-negative bacteria like, *Cupriavidusnecator,Alcaligeneslatus,* and *Escherichia coli* (Vandamme and Coenye, 2004).In this study, the Sudan Black staining method was followed, to confirm the PHB accumulation of each bacterial strain. When observed under phase-contrast microscopy, granules were found to fill the cells almost entirely. The granules were spherical to oval-shaped the results well confirmed with the findings of earlier workers Pal and Paul, 2002. Among the 32 strains the six strains, such as SCBT 03, SCBT 11, SCBT 13, SCBT 21, SCBT 24, and SCBT 28 are PHB positive (Fig. 2). It indicates the all the colonies isolated from the E2 mineral medium was not producing PHB, and a cloudy (or) turbid nature of colonies may also be due to the production of biopolymers other than PHBthe similar results were confirmed by (Kanmani and Jayalakshmi, 2007). To confirm the PHB production, the 6 potential strains were inoculated in E2 mineral broth and kept for incubation, after that a total biomass and degradable diester weight was estimated, based on their biopolymer content the strain SCBT 11 has shown maximum accumulation and it was selected for further study (Fig. 3).

Moreover, when testing with different salinity, 5% salinity showed maximum accumulation, and increase of salinity also reverse to PHB production (Fig. 4).Generally, in limited nitrogen sources, only the PHB production was high and these granules will act as a storage place for a carbon source. Anderson and Dawes (1990) and Brauneg *et al.* (1978) reported a high accumulation of PHB by *Alcaligenesfaecalis*using fructose as the carbon source. The use of yeast extract as the nitrogen source has been reported by Fukui (1976), Nishimura *et al.*,(1978), and Fernandez-Castillo *et al.* 1986 observed no considerable effect of vitamins on biomass and biodegradable polymer production was observed. But, in this present study, different carbon sources (such as Glucose, Lactose, Sucrose, Maltose, and Cellulose) and nitrogen sources ((NH4)2HPO4, KNO3, NH4Cl, Tryptone, and Peptone) at 0.3% were tested, in that sucrose and diammonium hydrogen phosphate showed maximum PHB production (Fig.5 and Fig. 6).

For mass scale culture, optimized E2 mineral broth was prepared and pseudomonas spp., of SCBT 11 was inoculated, incubated at 30ºC for 36hrs with the potential carbon and nitrogen sources. After the incubation period extraction was done with chloroform. Many researchers have reported the extraction of biodegradable polymer with chloroform from bacterial biomass (Anwar and Hakim, 2002). During extraction, the production of PHB was noticed by the formation of a thin layer. The extraction with chloroform may be due to the presence of PHB granules that interlinked with each other and form a thin layer (Senthilkumar and prabakaran, 2006). The obtained content of PHB fromSCBT 5111 (*Pseudomonas* spp.) was calculated. The total cell dry weight was 1.93g and the biodegradable polyester was 0.04g and the total biopolymer content was 2.07%.

Fig 1:THB population in different seaweed associated bacteria samples

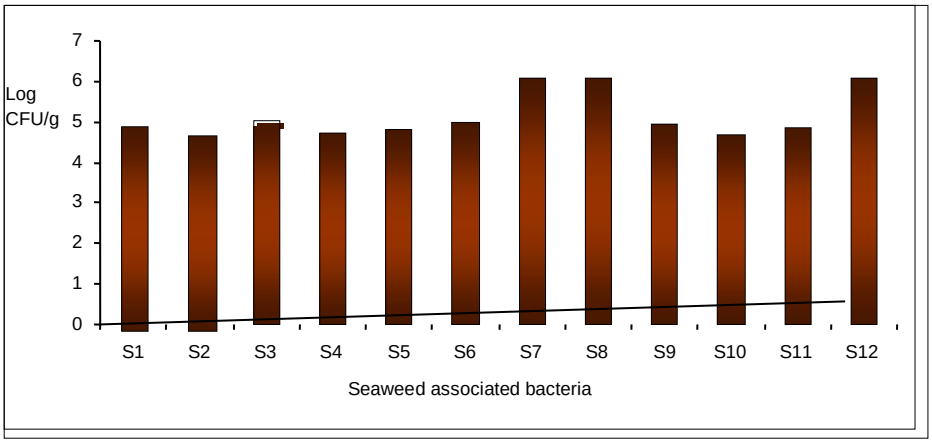


Table 1: Sudan Black staining and Gram Nature of the isolated bacterial strains

Strain Number	Sudan Black staining	Gram Nature
SCBT 01	PHB -	Negative
SCBT 02	PHB -a	Positive
SCBT 03	**PHB +**	**Negative**
SCBT 04	PHB -	Positive
SCBT 05	PHB -	Positive
SCBT 06	PHB -	Negative
SCBT 07	PHB -	Positive
SCBT 08	PHB -	Positive
SCBT 09	PHB -	Positive
SCBT 10	PHB -	Negative
SCBT 11	**PHB +**	**Negative**
SCBT 12	PHB -	Positive
SCBT 13	**PHB +**	**Negative**
SCBT 14	PHB -	Positive
SCBT 15	PHB -	Positive

SCBT 16	PHB -	Negative
SCBT 17	PHB -	Positive
SCBT 18	PHB -	Negative
SCBT 19	PHB -	Positive
SCBT 20	PHB -	Negative
SCBT 21+	PHB -	Negative
SCBT 22	PHB -	Negative
SCBT 23	PHB -	Negative
SCBT 24	**PHB +**	**Negative**
SCBT 25	PHB -	Positive
SCBT 26	**PHB +**	**Negative**
SCBT 27	PHB -	Positive
SCBT 28	**PHB +**	**Positive**
SCBT 29	PHB -	Positive
SCBT 30	PHB -	Negative
SCBT 31	PHB -	Positive
SCBT 32	PHB -	Positive

Fig 2: PHB accumulation of the bacterial strains

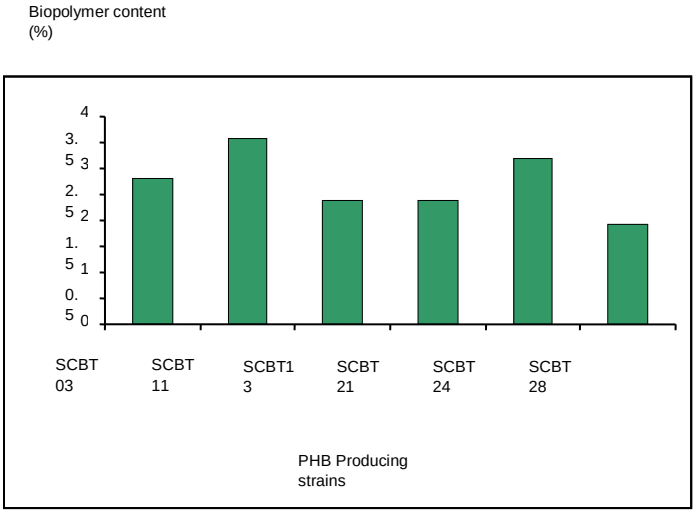


Fig 3: PHB production in the different incubation periods

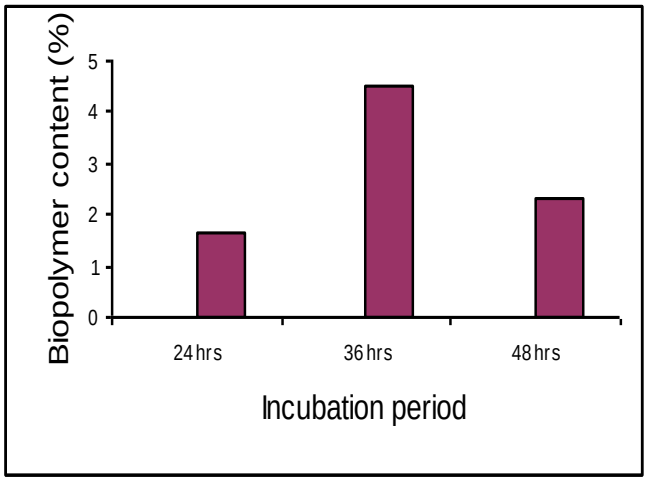


Fig 4: PHB production in different salinity

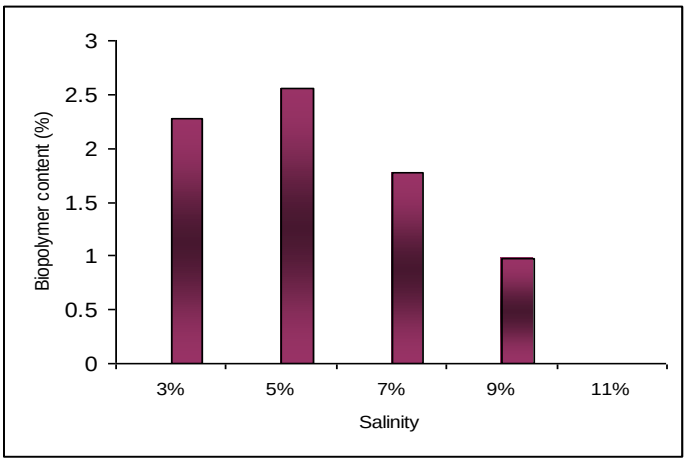


Fig 5: PHB production in different carbon sources

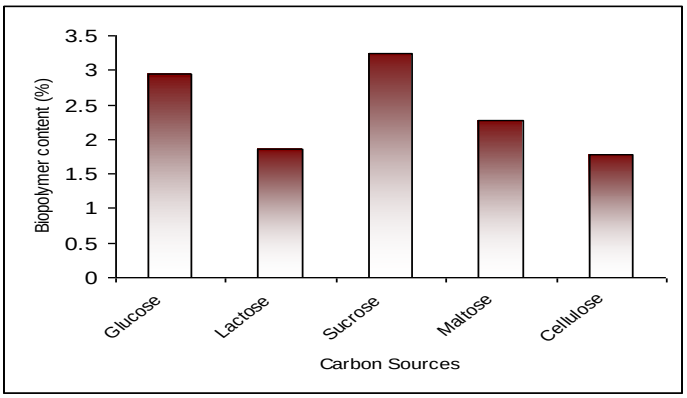
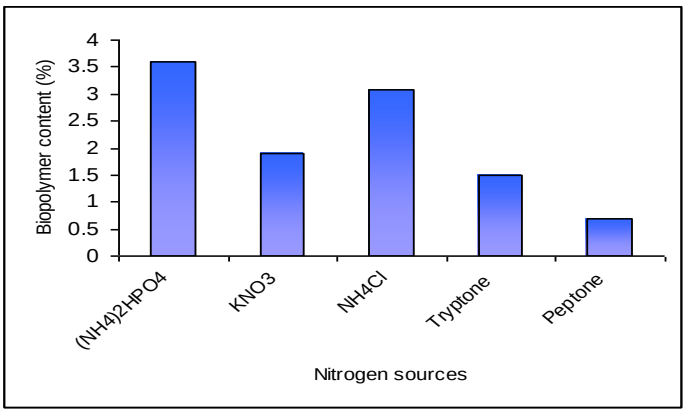


Fig 6: PHB production in different nitrogen source



Conclusion

From the results, the work highlighted the mass-scale production of biopolymer (poly (3-hydroxybutyrate) (PHB) from marine bacteria of SCBT 11 *Pseudomonas* spp. The results from the optimization maximum and minimum production of PHB production by using one parSCBTer at a one-time method had shown the potential temperature, salinity, pH, carbon, and nitrogen source for the production of biopolymer. Hence, this study demonstrated the production of biopolymer from marine bacteria, and the biopolymer are suggested to use and make as an eco-friendly biodegradable product.

Reference

1. Akiyama, M.; Tsuge, T.; Doi, Y. (2003). Environmental Life Cycle Comparison of Polyhydroxyalkanoates Produced from Renewable Carbon Resources by Bacterial Fermentation. Polym.Degrad. Stabil.,Vol. 80, pp 183–194.
2. Anderson, A.J., and Dawes, E.A.(1990). Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiology Reviews. Vol .54: pp 450-472.
3. Anwar,M.N. and M.A. Hakim, (2002). Isolation and characterization of poly–β–hydroxybutyrate producing bacteria. Bangladesh J. Microbiol. 19: 91–3.
4. Bonartseva, G.A., Myshkina, V.L. and Zagreba, E.D. (1994). Poly- hydroxybutyrate content in cells of various Rhizobium species during growth with different carbon and nitrogen sources. Microbiol., 63(1): 45-48.
5. Braunegg, G. B. Sonnleitner and R.M. Lafferty, (1978). A rapid gas chromatographic method for the determination of poly–β– hydroxybutyric acid in microbial biomass. European J. Appl. Microbiol. Biotechnol., 6: 29–37.
6. Braunegg, G.,Lefebvre, G.,Genser, K. (2003). Polyhydroxyalkanoates, Biopolyesters from Renewable Resources: Physiological and Engineering Aspects. J. Biotechnol. Vol. 65,pp127–161.

7. Cappuccino, J.G. and Sherman, N. (2002) Microbiology: A Laboratory Manual. 6th Edition, Pearson Education Inc., San Francisco, 15-224.
8. Chen.(2010). Plastics from Bacteria: Natural Functions and Applications, Microbiology Monographs. Vol.14,DOI10.1007/978-3-642-03287_5_7, © Springer-Verlag Berlin Heidelberg.
9. Fernandez–Castillo, R..V.F. Rodriguez., R.J. Gonzalez and B.F. Ruiz., (1986). Accumulation of poly (β– Hydroxybutyrate) by Halobacteria. Appl. Environ. Microbiol. 51: 214–6.
10. Fukui, T.A. Yoshimoto., M. Matsumoto, S. Hosokawa., T. Satio., H. Nishikawa and K. Tomita, (1976). Enzymatic synthesis of poly–β–hydroxyburtyrate in Zoogloea ramigera Arch. Microbiol. 110: 149– 56.
11. Jehan Mohamed El-Mohamedy Hawas., Tarek El-Said El-Banna. Elsayed Belal Abdelmonteleb Belal. and Ahmed AhmedAbd El-Aziz. (2016). Production of Bioplastic from some.selected.Bacterial strains. Int.J.Curr.Microbiol.App.Sci. 5(1):10-22. doi: http://dx.doi.org/10.20546/ijcmas.2016.501.002
12. Jobling, S., et al. (1995).A variety of environmentally persistent chemicals including some phthalate plasticizers are weakly estrogenic. Environ Health Perspect. Vol.103, pp. 582.
13. Kim, S. W., P. Kim., H. S. C Lee and J. H. Kim. (1996). High production of poly-beta-hydroxybutyrate (PHB) from Methylobacteriumorganophlium under potassium limitation. Biotechnol.Let. Vol.18pp25-30.
14. Kitamura, S., and Y. Doi. (1994). Staining method of poly (3-hydroxyalkanoic acids) producing bacteria by nileblue.Biotechnology. Vol.8pp 345-350.
15. Lauralynn Kourtz., Kevin Dillon., Sean Daughtry., Oliver P Peoples., Kristi D Snell. (2007). Chemically inducible expression of the PHB biosynthetic pathway in Arabidopsis. (6):759-69.PMID: 17279436. DOI: 10.1007/s11248-007-9067-1.
16. Lovejoy, T.E. Reaka-Kudla, M.K.,Wilson, D.E.,Wilson, E.O. (Eds.), (1997). Biodiversity: what is it? In: Biodiversity II: Understanding and Protectingour Biological Resources. Joseph Henry Press,Washington DC, pp. 7–14.
17. Madison, L.L. and G.W. Huisman,(1999).Metabolism of poly(3-hydroxyalkanoates): FromDNA to plastic.Microbiology.Vol.63,pp 21-53.
18. Nishimura, T.T. Saito and K. Tomita, (1978). Purification and properties of β–Ketothiolase from Zoogloea ramigera Arch. Microbiol. 116: 21– 7.
19. Norris J.R. , D.W. Ribbons. (1971). Methods in Microbiology. https://doi.org/10.1016/S0580-9517(08)70638-X Academic Press Inc. (London) Ltd. Published by Elsevier Ltd., Volume 5, Part B,Pages iii-vii, 1-695.
20. Ostle, A. G. and J.G. Holt. (1982).Nile blue A as a fluorescent stain for poly –beta- hydroxybutyrate. Applied Environmental Microbiology. Vol.44pp 238-341.
21. Poirier, Y., Nawrath, C. and Somerville, C (1995). Production of polyhydroxyalkanoates a family of Biodegradable plastics and elastomers in bacterial and plant. Biotechnol. 13: 142-150.
22. Reddy, C.S., Ghai, R., Rashmi, Kalia, V.C.(2003).Polyhydroxyalkanoates: an overview. Bioresource Technology. Vol. 87pp 137–146.
23. Schlegel, H.G., R. Lafferty and I. Krauss. (1970). The isolation of mutants not accumulating poly – beta hydroxybutricacid. Microbiology. Vol.71pp 283-294.
24. Senthilkumar and Prabakaran, (2006). Production of PHB (bio plastic) using bio- effluent as substrate by *Alcaligens eutrophus*. Indian journal of biotechnology. Vol5,pp 76- 79.

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