ISOLATION, CHARACTERIZATION OF POLY-HYDROXY BUTYRATE (PHB) PRODUCING BACTERIA FROM PADDY SOIL AND CONTAMINATED LAKE WATER AND COMPARATIVE STUDY FOR PRODUCTIVITY OF PHB FROM DIFFERENT ISOLATES

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ABSTRACT

Bio plastics are naturally occurring biodegradable polymers made from polyhydroxyalkanoates (PHA) of which poly 3-hydroxy butyric acid (PHB) is the most common. PHB serves as an energy storage molecule and accumulates intracellular as storage granules in microbes. This work utilized the biodiversity of bacteria to isolate various species from different environments and screen them for their ability to produce PHB. Two samples (Red soil from field and contaminated water from a lake) were collected, assayed and screened for PHB production. Six species were identified by Sudan Black screening methods positive for PHB production in natural conditions. Two different production medium were used such as a synthetic media (MSM media) and a complex media (Nutrient agar media) with addition of 1% glucose in both. Presence of accumulation of PHB in these strains was confirmed and estimated by Spectrophotometric analysis. *Micrococcus* and *Bacillus azotoformans* species isolated from soil sample showed maximum production of 0.162 mM and 0.157 mM respectively in modified Nutrient broth whereas *Pseudomonas* from water sample showed comparatively good production of 0.166 mM.

Keywords : Polyhydroxy-butyrate, Sudan black staining, Production Media, Spectrophotometric assay.

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Introduction

Most commercial plastics are synthetic polymers derived from petrochemicals. They tend to resist biodegradability. Petroleum based plastics are almost exclusively made from a non-renewable resource (petroleum), which is also the main source of energy for today's world, that mainly include polypropylene (PP), polyethylene (PE) and polystyrene (PS). (**Hans-Georg Elias, 2005**). In western world, a large amount of fossil carbon was used by chemical industries and a part of this is used for production of plastics and polymers every year. The growing piles of plastic waste constitute a severe environmental problem of soaring impact. **(Daniel D. Chiras 2004, Knight 2012).** Therefore, there is a need to study and to develop new biodegradable polymers with plastic-like properties. Polyhydroxy-alkanoates (PHAs) are biodegradable polyesters synthesized by microorganisms as carbon and energy storage materials under conditions of limiting nutrients such as nitrogen, phosphate or oxygen together with an excess of carbon source. (**Jacquel, N.; et al. 2008)**. Polyhydroxy-butyrate (PHB) is a linear polyester of D-3- hydroxyl-butyrate , and the best known of the polymers of related polyhydroxyalkanoates, that is produced by several microorganisms as an energy source. It is a biodegradable plastic that can be degraded aerobically and anaerobically by soil microorganisms. It has similar physical properties as polypropylene so it can be used as an alternative source of plastics. Polyhydroxy-butyrate (PHB) is a polyhydroxyalkanoate (PHA), a polymer belonging to the polyesters class that are of interest as bio-derived and biodegradable plastics. **(Yutaka Tokiwa; et al. September 2009)**.

Materials and Methodology

Sample Collection

The soil sample was collected from the paddy field from Ghatkeswar, Hyderabad and the water sample was collected from the contaminated lake of Safilguda, Secunderabad, Telengana State

Isolation of Bacteria

1 gm of soil sample was first added to 9ml of 0.89% Sodium Chloride solution and was serially diluted upto 10^{-7} dilutions. Likewise 1 ml of water sample was first added to 9ml of 0.89% Sodium Chloride solution and was serially diluted upto 10^{-7} dilutions. 0.1 ml of diluents was taken and was spread plated on Nutrient Agar Media (Peptone- 5gm, Sodium Chloride- 5 gm, Beef Extract- 3gm, Agar- 18gm, Distill Water- 1000 ml). The plated were then kept in the incubator for 24 hours at 37^0C .

Screening for isolates producing Polyhydroxy-butyrate (PHB)

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black Dye. After the staining with Sudan Black, bacteria containing PHAs exhibit dark granules. (**Burdon KL, 1946**) . The bacterial colonies were isolated and streak plated on Nutrient Agar Media (Peptone- 5gm/L, Sodium Chloride- 5gm/L , Beef Extract- 3gm/L, Agar- 18gm/L, Distill Water- 1000 ml) with 1% of glucose and kept for incubation at 37^0C for 24 hours. Then the ethanolic solution containing 0.02% Sudan Black B was spread over the Petri plates containing colonies and was kept undisturbed for 30 min. They were washed with 96% ethanol to remove excess stain from colonies

Identification of bacterial isolates producing Polyhydroxy-butyrate (PHB)

The different bacterial isolates that gave positive result to the Polyhydroxy-butyrate (PHB) production were then identified by staining and different biochemical methods as described in Bergey's manual for Determinative Bacteriology. (**Bergey, David H.; John G. Holt; Noel R. Krieg; Peter H.A. Sneath 1994).**

Various staining procedures such as gram staining (**Gram, HC 1884)**, Endospore staining (**Lechtman, M. D., et al., 1965**) and different biochemical tests that included Starch Hydrolysis (**Smibert, Robert M. and Noel R. Krieg. 1994)**, Catalase Test(**Maehly AC, Chance B 1954**)., Simmons citrate test **(Koser, S.A. (1923)**, Gelatin Hydrolysis **(Greene RA, Larks GG. 1955**) Voges Proskauer test **(MacFaddin, J. F. 1980)**, Mannitol Fermentation test **(Hugh R, Leifson E. 1953).** were done to confirm the bacterial isolates.

Production for Polyhydroxy –butyrate

Two production media were prepared for production of Polyhydroxy-butyrate. One production media was modified Nutrient broth (Peptone- 5gm, Sodium Chloride- 5 gm, Beef Extract- 3gm, Distill Water- 1000 ml) with 1% glucose. The other media was Minimal Salt Medium (MSM)($Na_2HPO_4.2H_2O$- 4.5 gm/L, KH_2PO_4- 1.5gm/L, NaCl- 1.0 gm/L, $(NH_4)_2SO_4$- 2.0 gm/L, $CaCl_2.2H_2O$- 0.02 gm/L, NH_4Fe(III) citrate- 0.05 gm/L, $MgSO_4$- 0.2 gm/L, trace element solution*- 1ml/L, Glucose- 10 gm/L)(* trace element solution - $ZnSO_4.7H_2O$- 100 mg/L, H_3PO_4- 300 mg/L, $CoCl_2$- 200 mg/L, $CuSO_4$- 6 mg/L , $NiCl_2$- 20 mg/L, Na_2MoO_4- 30 mg/L, M_2Cl_2- 25 mg/L, the trace elements solution was prepared separately, autoclaved and mixed with the media.).

The bacterial isolates showing positive in the screening test for production of Polyhydroxy Butyrate were then inoculated into the production media and were kept for 72 hours at 37°C.

Extraction of Polyhydroxy –butyrate

Then the culture was collected and centrifuged at 8000 rpm for 15 minutes at 4^0C. The supernatant was discarded and the pellet was treated with 10 mL sodium hypochlorite and incubated at 30°C for 2h. After incubation, the mixture was centrifuged at 8000 rpm for 10minutes at 4^0C and washed sequentially with sterile distilled water, acetone and alcohol. Finally the residue was extracted with boiling chloroform and poured in a sterile glass petri plate for evaporation of chloroform. After all the chloroform is evaporated the extract is stored in -20°c for further analysis. (**Arnold L, Demain J, Davis E. 1999**).

Estimation of PHB produced

a - Dry Weight Estimation

The correct estimation on dry weight is very important in determining the amount of PHB produced by each strain. The sample extracted was filtered through pre-wetted Whatmann No.1 filter paper. The chloroform extract was evaporated to dryness. Determination of PHB yield was performed by dry weight estimation. The dried PHB was weighed using a calibrated digital

weighing scale. (**Zakaria M. R, Ariffin H, Johar N. A. M, Aziz S. A, Nishida H, Shirai Y, Hassan M. A. , 2010**)

b - Colorimetric Estimation (Jhon and Ralph (1961))

The UV absorption spectrum of PHB was analyzed following its conversion to crotonic acid by treatment with conc. H_2SO_4. The formation of crotonic acid gives a brown colour to the solution which can be significantly used to measure concentration depending on the intensity of the colour. Sample was transferred into a clean test tube and 10ml of concentrated H_2SO_4 was added to test tubes and it was capped. The capped test tubes were heated for 10 min at 100°C in water bath. The solution was cooled and after thorough mixing a sample was transferred to silica cuvette and the absorbance was checked at 445nm. The absorbance was measured against sulphuric acid as blank.

Results

The plates that were incubated for 24 hours after spread plating of the water and soil sample, Ten (10) number of colonies were isolated from the soil sample and Seven (7) number of colonies were isolated from water sample. The samples were named for water sample as S1, S2, S3, ….S10 and the water samples were W1, W2, ….., W7. The colonies were first identified by colony morphology. The Sudan black staining screening test is used to screen for the production of Polyhydroxy Butyrate (PHB) by the bacterial isolates.

The bacterial isolates such as S2, S3, S4, S9, W1 and W7 had shown positive for the production of Polyhydroxy Butyrate (PHB). (Figure 1 – Figure 4).

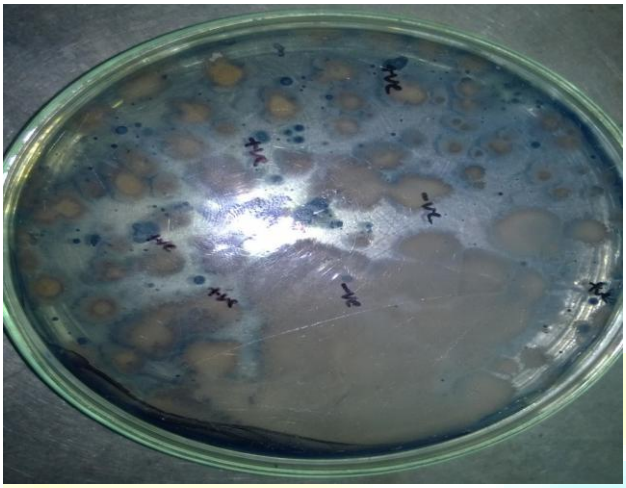


Figure 1 : primary screening of master plate.

Figure 2 : Sudan Black staining for S2, S3, W1

Figure 3 : Sudan Black staining for S4, S9 result whereas S6 gave negative.

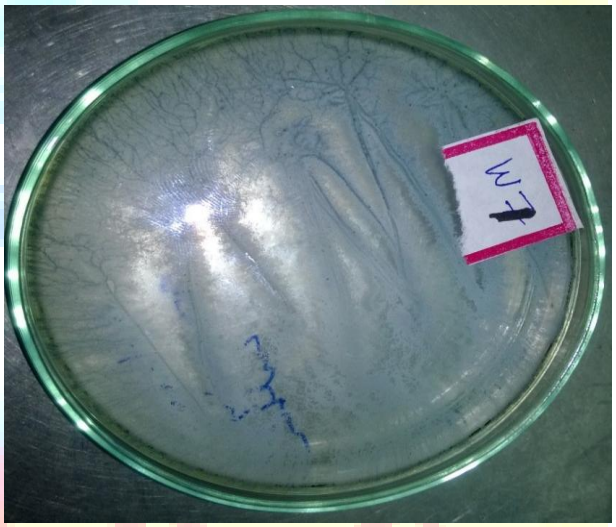


Figure 4 : Sudan Black staining for W7.

Those different strains S2, S3, S4, S9, W1 and W7 were subjected to staining procedures and different biochemical tests and their results are described in the Table no : 1 , Table : 2.

Table 1 : Gram Staining results of S2, S3, S4, S9, W1 and W7 ; Endospore staining of S9 and W1.

Sample	Strain	Gram Staining	Morphology	Endospore Staining
Soil Sample	S2	Positive	Steptococcus, small spherical	NA
	S3	Positive	Elongated rods and chained bacillus	NA
	S4	Positive	Bacillus, unchained	NA
	S9	Positive	Rod shaped	Negative
Water Sample	W1	Positive	Bacillus chained	Positive
	W7	Negative	Rod shaped cocci bacillus	NA

NA- not achieved

Table 2: Results of biochemical tests of S2, S3, S4, S9, W1 and W7.

Sample	Strain	Catalase test	Starch hydrolysis	Simon citrate	VP test	Manitol test	Glucose test	Gelatin hydrolysis
Soil Sample	S2	Positive	-	-	-	-	-	-
	S3	Positive	Positive	Negative	Positive	Negative	-	-
	S4	Negative	Negative	Positive	-	-	-	-
	S9	Positive	Negative	-	-	-	Negative	-
Water Sample	W1	Negative	Negative	Negative	-	-	-	Positive
	W7	Negative	Negative	Negative	-	-	Negative	-

Thus basing upon the staining and their activity towards the different biochemical test, the different bacterial isolated were described in Table 3.

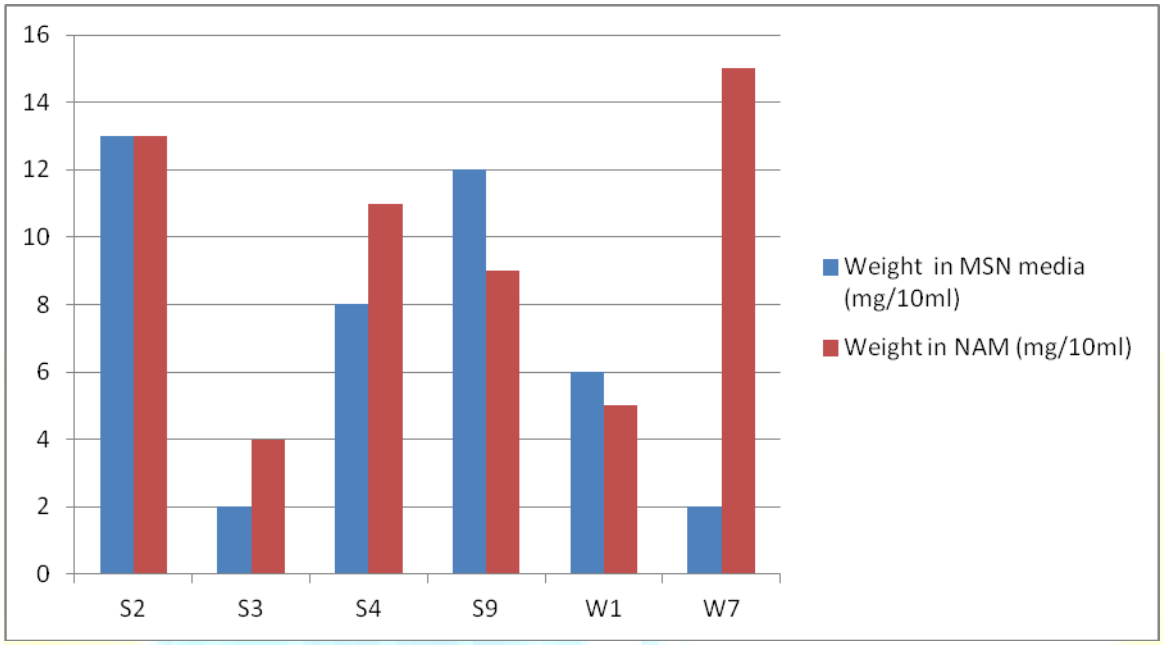
Table: 3: Identified bacterial isolates.

Sample	Strain	Name
Soil Sample	S2	*Micrococcus*
	S3	*Bacillus alvei*
	S4	*Bacillus azotoformans*
	S9	*Bacillus marinus*
Water Sample	W1	*Bacillus popillae*
	W7	*Pseudomonas*

The PHB production was first estimated by dry weight estimation method from both Nutrient production broth (NAM) and the Minimal Salt medium (MSN) broth.(Table 4)(Graph 1).

Table 4: Dry weight estimation of PHB produced by different organisms in different production media.

Sample	Weight in MSN media (mg/10ml)	Weight in Nutrient agar media (mg/10ml)
Micrococcus [S2]	13mg	13mg
Bacillus alvei [S3]	2mg	4mg
Bacillus azotoformans [S4]	8mg	11mg
Bacillus marinus [S9]	12mg	9mg
Bacillus popillae [W1]	6mg	5mg
Pseudomonas [W7]	2mg	15mg

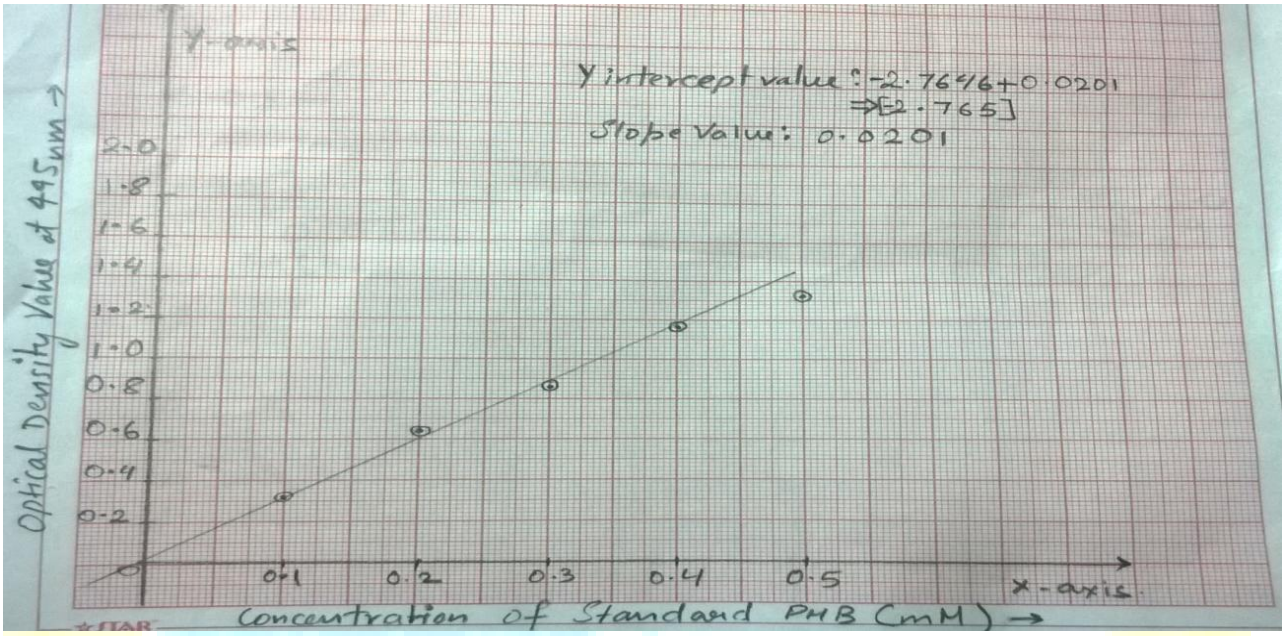


Graph 1 : Graphical representation of PHB production (in mg/mL) by different isolates in different production media

The second estimation was done by colorimetric method at 445nm. The concentration of PHB was found in mM. The molecular weight of the PHB has not been determined as it varies with the different species and conditions of production. Standard Graph was obtained by using the known concentration of PHB and the concentration of PHB produced was found with the formula

Formula:

$$\beta - Hydroxybutyrate\,(mM) = \frac{[corrected\ absorbance - y\ intercept]}{slope} \times Dilution$$

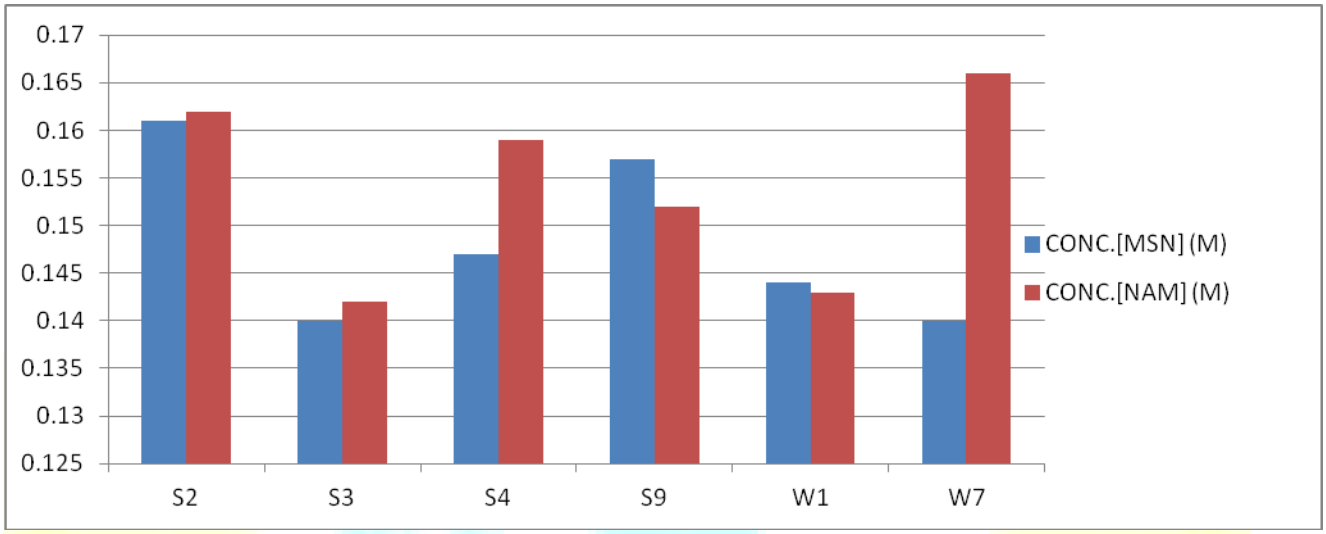


Graph 2: Standard graph for PHB.

The unknown concentration of the PHB produced is determined by plotting the Optical Density/absorbance at 445 nm in the standard graph of PHB (Table: 5)(Graph : 3).

Table 5: Different concentration of PHB produced by different isolates in different production media.

SAMPLE	OD(MSN)	CONC. (mM)	CONC. (M)	OD (NAM)	CONC (mM)	CONC. (M)
Micrococcus [S2]	0.49	161.94	0.161	0.51	162.93	0.162
Bacillus alvei [S3]	0.05	140.04	0.140	0.10	142.53	0.142
Bacillus azotoformans [S4]	0.20	147.51	0.147	0.45	159.91	0.159
Bacillus marinus [S9]	0.40	157.46	0.157	0.30	152.49	0.152
Bacillus popillae [W1]	0.13	144.02	0.144	0.11	143.03	0.143
Pseudomonas [W7]	0.05	140.04	0.140	0.58	166.41	0.166



Graph 3: Graphical representation of PHB production(in Molar) by different isolates in different production media**s.**

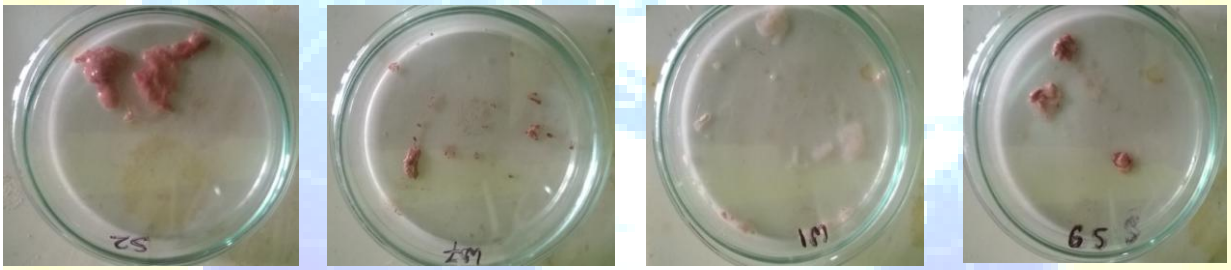


Figure 5 : Production of PHB by different strains of Bacterial isolates.

Discussion

Micrococcus[S2], Bacillus Alvei[S3], Bacillus azotoformans[S4], Bacillus marinus[S9], were isolated from soil sample collected from paddy field and *Bacillus popillae[W1], and Pseudomonas[W7]* were isolated from contaminated lake water sample were able to produce PHB. Two different types of medias were used for production in this study, Nutient Broth with 1% glucose (complex media) showed more amount of PHB accumulation than Minimal Salt Media with 1% glucose (Synthetic medium). *Pseudomonas* [W7] from water sample produced 0.166M of PHB in NAM and 0.140M in MSN media. Bacteria have been found to accumulate PHBs when there is a limitation of nutrients, specifically nitrogen sources (**Steinbuchel and Fuchtenbusch, 1998**) and excess of carbon source. Isolation of new strains capable of utilizing the cheap carbon source is essential to reduce the cost at industrial level (**Steinbuchel, 1998**).

Bacillus sp are known for their rapid growth on simple nutrients. Earlier, researches have focused their studies on the production of PHAs using specialized media. Bacteria which are able to produce PHB in using complex starch sources are rarely documented. Among the strains produced the maximum production was shown by *Pseudomonas [W1](15mg/10ml)* from water sample followed by *Micrococcus* [S2](13mg/ml), *Bacillus marinus* [S9](12mg/ml) and *Bacillus azotoformans* [S4](11mg/ml). Other two species *Bacillus popillae* [W1](6mg/ml) and *Bacillus alvei* [S3](4mg/ml) showed relatively less production.

Conclusion

Maximum production of PHB was obtained by *Pseudomonas* and *Micrococcus*. Two production medium were compared for the PHB production and the modified nutrient broth was given better production for more strains as compared to Minimal salt medium. High production costs have not enabled the full commercialization of bioplastics although, a number of strategies such as the use of recombinant microbial strains, more efficient fermentation processes and recovery/purification protocols and the use of inexpensive substrates have been adopted to bring down the production costs. Further studies can be done by optimizing the production media to minimize the cost of production and also recombinant strains can be made to increase the level of production which would help in commercialization of this bioplastic in near future.

References

1. A.P. Bonartsev, V.L. Myshkina, D.A. Nikolaeva, E.K. Furina, T.A. Makhina, V.A. Livshits1, A.P. Boskhomdzhiev, E.A. Ivanov, A.L. Iordanskii, and G.A. Bonartseva, “Biosynthesis, biodegradation, and application of poly(3-hydroxybutyrate) and its copolymers - natural polyesters produced by diazotrophic bacteria”, A. Méndez-Vilas (Ed), 2007.
2. Aarthi.N, Ramana.K.V, “dentification and Characterization of Polyhydroxybutyrate producing Bacillus cereus and Bacillus mycoides strains”, International Journal Of Environmental Sciences, Volume 1, No 5, 2011

3. Alejandra Rodriguez-Contreras1, Martin Koller, Miguel Miranda-de Sousa Dias, Margarita Calafell, Gehart Braunegg and María Soledad Marqués-Calvo, "Novel Poly[(R)-3-Hydroxybutyrate]-Producing Bacterium Isolated from a Bolivian Hypersaline Lake", FTB-2926, September 6, 2011
4. Arnold L, Demain J, Davis E. Polyhydroxyalkanoates. Manual of Microbiology and Biotechnology. Washington, Am Soc. Microbiol. 1999; 2: 616-627.
5. B Senthil Kumar and G Prabakaran, "Production of PHB (bioplastics) using bio-effluent as substrate by Alcaligens eutrophus", Indian Journal of Biotechnology Vol. 5, January 2006.
6. Bergey, David H.; John G. Holt; Noel R. Krieg; Peter H.A. Sneath (1994).*Bergey's Manual of Determinative Bacteriology* (9th ed.). Lippincott Williams & Wilkins.
7. Burdon KL. Fatty materials in bacteria and fungi revealed by staining dried, fixed slide preparations. J. Bacteriol. 1946;52:665–678
8. Daniel D. Chiras (2004). Environmental Science: Creating a Sustainable Future. Jones & Bartlett Learning. pp. 517-518.
9. Gram, HC (1884). "Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten". *Fortschritte der Medizin* (in German) **2**: 185–189.
10. **Greene RA, Larks GG.** 1955. A quick method for the detection of gelatin liquefying bacteria. J. Bacteriol. **69:**224
11. Gulab Singh, Arpana Mittal, Anish Kumari, Varsha Goel, Neeraj K Aggarwal and Anita Yadav, "Optimization of Poly-B-Hydroxybutyrate Production from Bacillus species" , European Journal of Biological Sciences 3 (4): 112-116, 2011
12. **Hugh R, Leifson E.** 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J. Bacteriol. **66:**24–26
13. Hans-Georg Elias "Plastics, General Survey" in Ullmann's Encyclopedia of Industrial Chemistry, 2005, Wiley-VCH, Weinheim.
14. Jacquel, N.; et al. (2008). "Isolation and purification of bacterial poly(3-hydroxyalkanoates)". *Biochem. Eng. J.* **39** (1): 15–27
15. Koser, S.A. (1923). "Utilization of the salts of organic acids by the colon-aerogenes group.". *Journal of Bacteriology* **8**: 493–520

16. **Lechtman, M. D., J. W. Bartholomew, A. Phillips, and M. Russo.** 1965. Rapid methods of staining bacterial spores at room temperature. J. Bact. **89:**848–854.
17. MacFaddin, J. F. 1980. Biochemical Tests for Identification of Medical Bacteria, 2nd ed. Williams and Wilkins, Baltimore
18. Maehly AC, Chance B (1954). "The assay of catalases and peroxidases".*Methods Biochem Anal*. Methods of Biochemical Analysis **1**: 357–424.
19. Mustafa Kansiz, Helen Billman-Jacobe, Don Mcnaughton, "Quantitative Determination of the Biodegradable Polymer Poly(-hydroxybutyrate) in a Recombinant Escherichia coli", Applied And Environmental Microbiology, Vol. 66, Aug. 2000.
20. Paramjit Singh and Nitika Parmar, "Isolation and characterization of two novel polyhydroxybutyrate (PHB)-producing bacteria", ISSN 1684–5315 © 2011 Academic Journals.Steinbuchel A, Fuchtenbusch B (1998). Bacterial and other biological systems for polyester production. Trends Biotechnol. 16: 419-427.
21. Smibert, Robert M. and Noel R. Krieg. 1994. Page 630 in Methods for General and Molecular Bacteriology, edited by Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg,American Society for Microbiology, Washington, DC.
22. Sunitha Panigrahi, Ujwala Badveli, "Screening, Isolation and Quantification of PHB-Producing Soil Bacteria", International Journal of Engineering Science Invention, September. 2013.
23. Verlinden RAJ, Hill DJ, Kenward MA, William CD, Radeckal (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. J. Appl. Microbiol. Rev. 102: 1437-1449.
24. Yutaka Tokiwa; Buenaventurada P. Calabia; Charles U. Ugwu; Seiichi Aiba (September 2009). "Biodegradability of Plastics". *International Journal of Molecular Science***9**: 3722–3742.
25. Zakaria M. R, Ariffin H, Johar N. A. M, Aziz S. A, Nishida H, Shirai Y, Hassan M. A. Biosynthesis and characterization of poly (3-hydroxybutyrate-co-3-hydroxybutyrate) copolymer from wild type Comamonas sp. EB172. Polym. Degrad. Stab. 2010; 95: 1382-1386.