Research Article

Production, Optimization and Characterization of Polyhydroxybutryate by Bacillus subtilis Isolated from Garden Soil

E. Gayathiri, B. Bharathi, N. Siva, R. Prabavathi and S.Velu

Department of Plant Biology and Biotechnology, G. S. Gill Research Institute, Guru Nanak College, Chennai, Tamil Nadu, India.

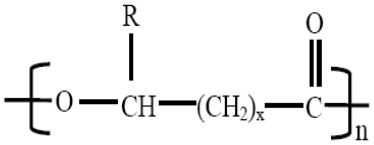
ABSTRACT

Polyhydroxyalkanoates (PHAs) are biopolymers stored by a wide variety of organisms as an energy reserve. They are polyesters formed by polycondensation of carboxylic acids with hydroxyl alcohol. Poly-3-hydroxybutyrate P(3HB), the simplest PHA, The biosynthesis of PHAs by a number of organisms occurs when these store energy in the form of polyester granules. Extensive studies and results have shown the flexibility and the ability of prokaryotic organisms to incorporate diverse substrates and consequently to produce different kind of biopolyesters. Polyhydroxybutyrate are naturally formed macromolecules during the growing cycle of the organisms, and therefore are referred to as natural polymers. Polyhydroxybutyrate (PHBs) play important roles in the attachment of bacterial cells to a surface and in building and maintaining the three-dimensional, complex structure of bacterial biofilms. Poly-3-hydroxybutyrate producing bacteria were isolated from different garden soil samples. Later Screening of bacterial strain for Poly-3-hydroxybutyrate production were done with many parameters. A bacterial strain that produces amylase and polyhydroxyalkanoate (PHA) was isolated, identified, and classified. The present study provide the useful data about the optimized conditions for PHB production by *Bacillus species* that can be utilized for industrial production of PHB, a fast emerging alternative of non biodegradable plastics.

Keywords: Polyhydroxyalkanoate, Polyhydroxybutyrate, *Bacillus subtilis*

INTRODUCTION

The annual world production of synthetic polymers amounts to about 140 million tons. A vast majority of this volume is composed of chemically stable polymers that are not easily degraded. These are mostly synthetic polymers produced essentially by chemical addition or condensation reactions in which a large number of monomers are joined sequentially. Biopolymers are naturally occurring materials: most materials formed in nature during the life cycles of green plants, animals, bacteria and fungi are polymers or polymer matrix composites. Biopolymers include the polysaccharides such as cellulose, starch, the carbohydrate polymers produced by bacteria and fungi and animal protein-based biopolymers such as wool, silk, gelatin and collagen. Biopolymers, especially the fibrous ones, have been used by mankind from the earliest days of civilization.



		Type of monomer
x = 1	R = methyl	3-hydroxybuytrate; 3HB
	R = ethyl	3-hydroxyvalerate; 3HV
	R = propyl	3-hydroxyhexanoate; 3HHx
x = 2	R = hydrogen	4-hydroxybutyrate; 4HB
x = 3	R = hydrogen	5-hydroxyvalerate; 5HV

Table – 1: CLASSIFICATION OF MICROBIAL BIOPLASTICS ACCORDING TO DIFFERENT CRITERIA (Jose *et al.*, 2003)	
Biosynthetic origin	Natural bioplastics: those produced by microorganisms from general metabolites (i.e. PHBs and aliphatic PHAs).
	Semisynthetic bioplastics: those that require the addition to the culture broth of some precursors that cannot be synthesised by the microbe (i.e. PHAs containing aromatic monomers)
	Synthetic bioplastics: those polyesters that resemble the natural ones but that can only be obtained by chemical synthesis (i.e. synthetic thermoplastic polymers)
Chemical nature of the monomers	Bioplastic containing aliphatic fatty acid derivatives: saturated or unsaturated (with double or triple bonds) monomers; linear or branched monomers; substituted or not (with functional groups in the monomers).
	Bioplastics containing aromatic fatty acid derivatives
	Bioplastics containing both aliphatic and aromatic fatty acid derivatives
	Bioplastics containing other different compounds (e.g. poly-g-glutamic acid, poly-e-L-lysine, poly-b-L-malic acid, polyglycolic acid, cianophicin)
Monomer size	Bioplastics containing a short-chain length (sclPHB and derivatives sclPHAs; C3–C5 monomers)
	Bioplastics containing a medium-chain length (mclPHAs; C6–C14)
	Bioplastics containing a long-chain length (lclPHAs; >C14)
Number of monomers in the polyesters	Homopolymericbioplastic: a single monomer is present in the bioplastic
	Heteropolymericbioplastic (copolymer): more than one monomer is present in the bioplastic
Type of polyesters accumulated by the microbe	Unique (a single bioplastic)
	More than one (mixed bioplastics)

Table-2: The role of Polyhydroxyalkanoates in bacterial environmental fitness. (Guo-Qiang Chen, 2010)
Cell survival under stressful low nutrient conditions
Cell survival under nutrient limitation in water, soil, rhizosphere, and phyllosphere
Cell survival in inoculant carriers
Establishment of inoculum in soil and plant surfaces
Energy source and flow for cell motility, chemotaxis, aerotaxis, and biological nitrogen fixation
Sporulation, cyst formation, and germination
Control of exopolysaccharide production
Endurance under environmental stress: heat and cold, UV irradiation, desiccation, osmotic and solvent stress, osmotic shock, ethanol, and H_2O_2
Balanced use of available energy and distribution of carbon resources

Polyhydroxyalkanoates (PHA), a family of biopolyesters with diverse structures, are the only bioplastics completely synthesized by microorganisms. PHA can be synthesized by over 30% of soil-inhabiting bacteria (Wu *et al.,* 2000). PHAs are polymers of carbon, oxygen and hydrogen and the general structure of the polymer is shown in Figure 1.

NATURAL FUNCTIONS OF BACTERIAL POLYHYDROXYALKANOATES

POLY-3-HYDROXYBUTYRATE

P(3HB) is the first member of PHAs to be discovered in microorganisms [Figure -2]. The role of P(3HB) as an energy source during nutrient-depletion for enhancing the survival of bacteria is well accepted. Today, it is known that P[3HB] is the most common PHA found in nature. Based on the MW of the biosynthesized P[3HB], they can be divided into three distinct groups, i.e., low MW P[3HB], high MW P[3HB], and ultra-high molecular weight (UHMW) P[3HB].

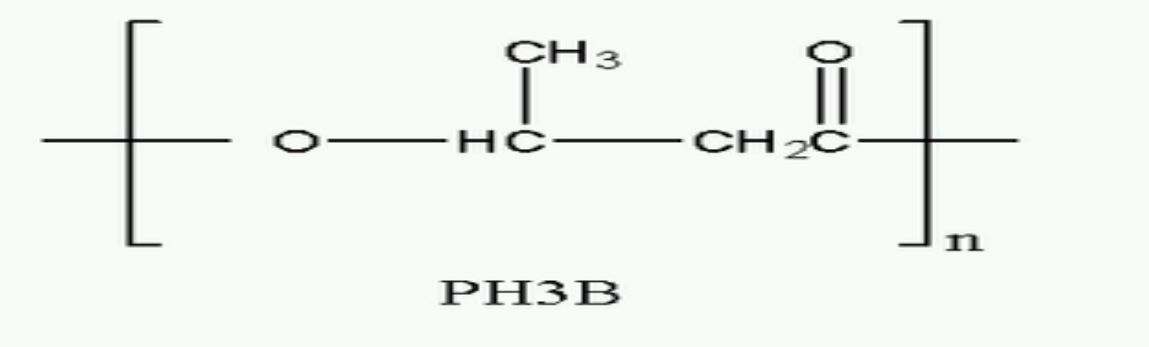


Figure 2: Chemical structure of poly [*R*-3-hydroxy butyrate] (P[3HB])

n = 120-200: Low molecular weight P[3HB]
n = 1,000-20,000: High molecular weight P[3HB]
n = 100,000: Ultrahigh molecular weight P[3HB]

MORPHOLOGY OF POLY-3-HYDROXYBUTYRATEGRANULES

PHB and other PHAs accumulate in discrete spherical granules in the cell cytoplasm. Granules have a diameter in the range between 100 and 800 nm and are enclosed in a unit membrane approximately 2-4 nm thick. The granules are typically composed of 98% polymer and 2% protein.

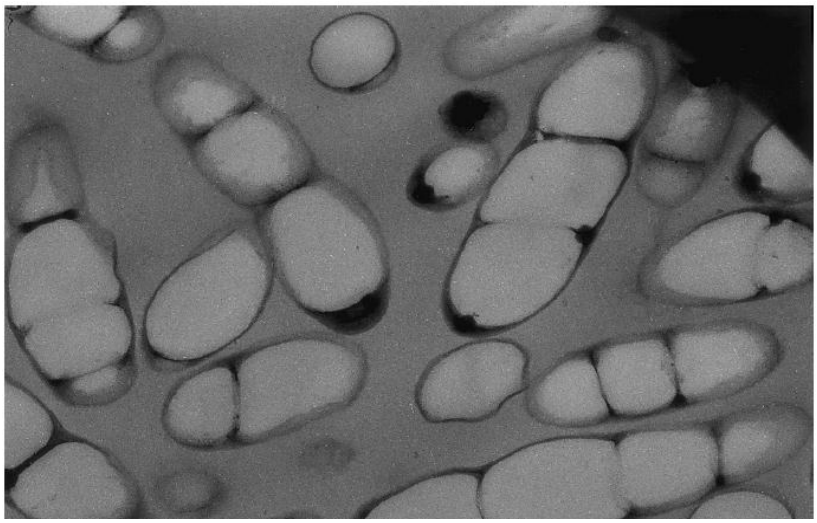


Fig. 3: TEM of thin sections of recombinant R. eutropha PHB24 cells containing large amounts P(3HB-co-5 mol% 3HHx). Bar represents 0.5 mm. (Sudesh *et al.*, 2000)

A comparison of PHB properties with that of other polymers is summarized in Table 3.

Table - 3: Properties of some PHAs and synthetic polymers (Lee 1996; Sudesh *et al.*, 2000)

Polymer	Melting Temperature °C	Glass-transition Temperature °C	Young's modulus GPA	Elongation To break %	Tensile Strength MPa
P(3HB)	180	4	3.5	5	40
P(3HB-co-3HV)*	145	-1	-	-	32
P(4HB)	53	-48	149	1000	104
PHA_{MCL}	45-54	-25 to -40	-	~ 350	≤17
Polypropylene	176	-10	1.7	400	34.5
Polystyrene	240	100	3.1	-	50

*** P(HB-HV) copolymer containing 20 mol% C5 and 80 mol% C4 monomer**

The aim of the present work was selection of Polyhydroxybutyrate producing *Bacillus spp*.

MATERIALS AND METHODS

The bacteria involved in this study were isolated from different garden soil samples present in Chennai. A total of 15 samples were collected and transported to laboratory for further processing. Samples were pretreated by heating at 60°C for 60 min in water bath and then serial dilution technique was performed for the isolation of different endospore forming bacteria and screened for the presence Polyhydroxybutyrate granules. Bacterial strains were confirmed by using endospore staining (MiracYilmaz *et al.,* 2005)

The endospore producing pure strains were screened for the presence of Polyhydroxybutyrate granules using Sudan B black staining technique (Das *et al.,* 2004). The positive cultures showing dark purple colour polyhydroxybutyrate granules were maintained separately in nutrient agar slants and used for further studies. The positive strain that produces maximum Polyhydroxybutyrate was selected and was given for identification in IBMS, University of Madras, Taramani. The selected bacterial isolates were used for further study.

POLYHYDROXYBUTYRATE PRODUCTION

The positive isolates showing PHB granules were inoculatedin Luria Bertani medium. The fresh overnight culture was used as an inoculum for production of polyhydroxybutyrate.The spectrometric chemical assay for the determination of PHB from the sample was estimated using Law and Splepecky method (Law and Splepecky, 1961).Standard Chart of PHB was estimated by Law and Splepecky, (1961). Estimation of PHB in sample were studied using Alkaline digestion method, (Nisha *et al.,* 2009).

The time course of production of PHB during fermentation, influence of various different pH levels, influence of different carbon and nitrogen sources on their overall production were investigated using Nutrient broth production medium (Nur *et al.,* 2004).Production of PHB from Agro products were performed with various natural agro products like rice bran, wheat bran and sugarcane bagasse and whey waste water from dairy industry. (Nur *et al.,* 2004). Isolation of Bacterial Genomic DNA was carried out by standard procedure technique.

RESULTS

In this study, a total of 24 bacterial strains were isolated from different soil samples collected in Chennai.

SCREENING FOR POLYHYDROXYBUTYRATE (PHB)

The isolates were screened for Polyhydroxybutyrate granules using Sudan Black B staining technique. Among the 24 strains, PHB granules were observed in 11 strains. The strains gave positive results for Sudan black staining was utilized for furthers studies.

ISOLATION OF BEST POLYHYDROXYBUTYRATE PRODUCER

The selected 11 isolates were inoculated in 3 different production medium for identifying the best Polyhydroxybutyrate producer. Among the 11 isolates the best Polyhydroxybutyrate producer was selected and subjected for further optimization studies. Among the three medium used, Modified Nutrient broth (**Nur *et al.,* 2004**) was selected for further studies.

IDENTIFICATION OF BACTERIA

The identification study showed that the isolated positive strain has been identified as *Bacillus subtilis* **(**Identification done by Dept. of Microbiology, IBMS, University of Madras, Taramani.)

CHEMICAL ASSAY

The Polyhydroxybutyrate was estimated by using Law and Splepecky method (Law and Splepecky, 1961).

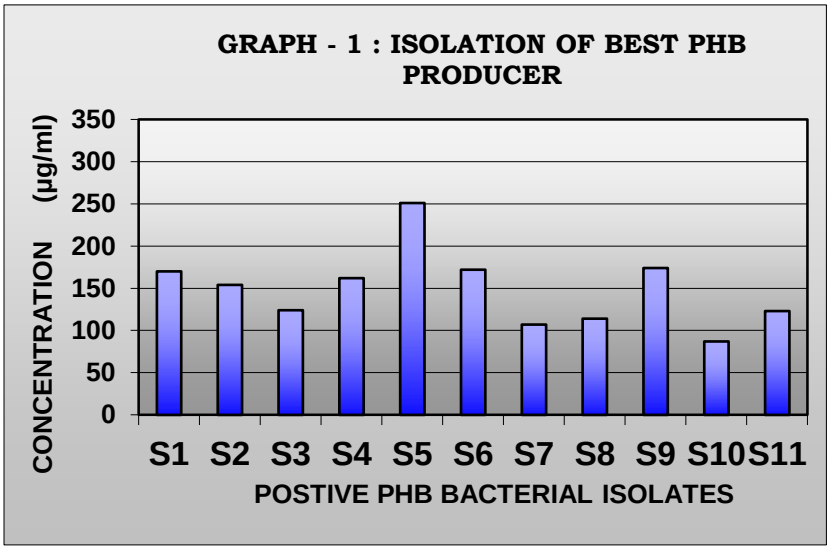
GROWTH STUDY

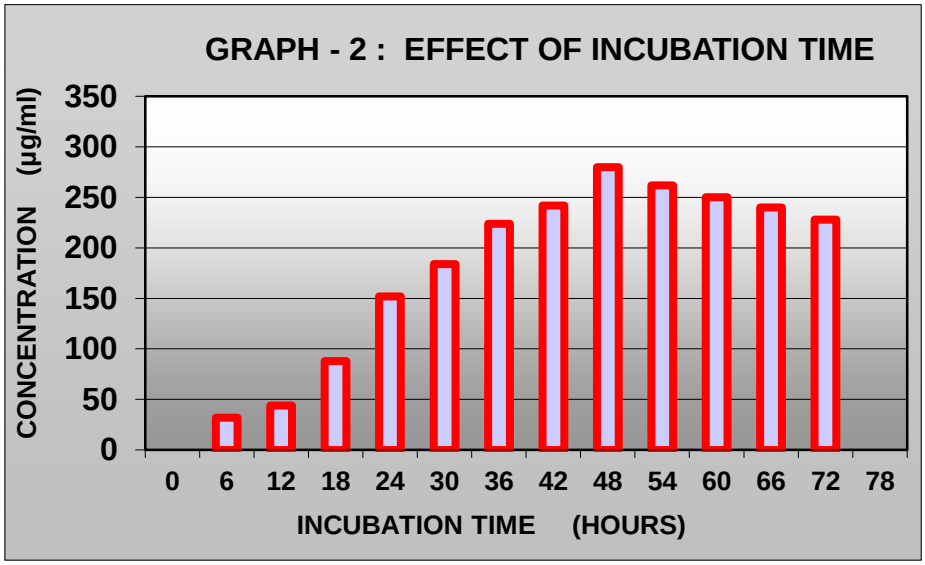
The growth study of the organism is essential for the production of Polyhydroxybutyrate. Growth study was performed for the selected isolate using modified nutrient medium **(Nur *et al.,* 2004)**. In order to determine the optimum production time for maximum Polyhydroxybutyrate production, the samples were collected at 6 hours intervals and analyzed for the estimation of Polyhydroxybutyrate. In the growth study

we found that upto 18th hour the production of PHB was very low and then there is a gradual increase in the production. Maximum PHB production was observed from 36th hour to 60th hour; from there onwards gradual decrease in the PHB production was observed. Based on the results analyzed at different time intervals, it was determined that the maximum production of PHB was at 24th hour. (Table 2 & Graph 1, 2).

Table – 2: Effect of Incubation Time on PHB Production

S. No.	Time of culture withdraw	PHB (µg/ ml)
1	6th hour	32
2	12th hour	44
3	18th hour	88
4	24th hour	152
5	30th hour	184
6	36th hour	224
7	42th hour	242
8	48th hour	280
9	54th hour	262
10	60th hour	250
11	66th hour	240
12	72th hour	228





Different Parameters

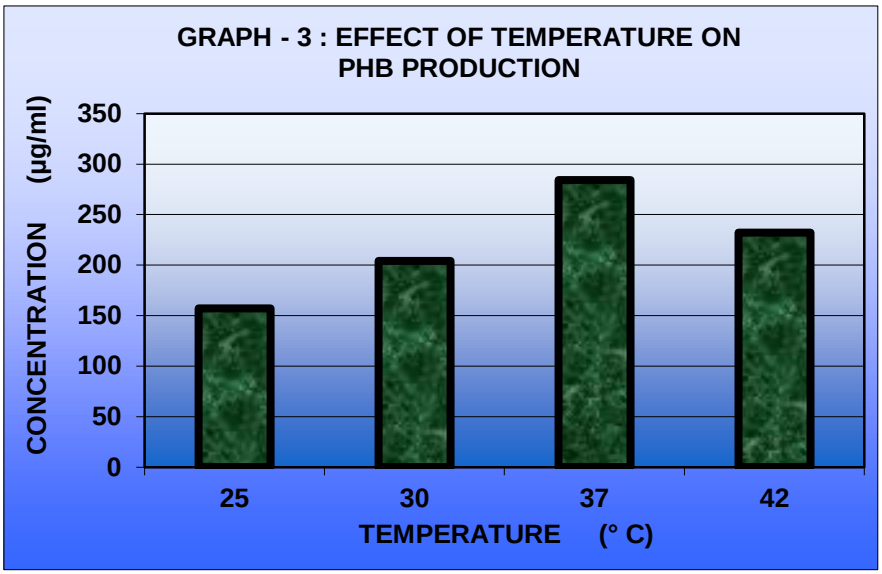
The environmental parameters like pH, temperature shows great influence on the growth of the organisms and the production of Polyhydroxybutyrate.

Effect of Temperature

In order to determine the effect of the incubation temperature for the better Polyhydroxybutyrate production, different incubation temperatures were maintained for production process. Based on the readings it was observed that the selected strain have a temperature optima at 37°C. (Table 3 & Graph 3).

Table – 3: Effect of Temperature on PHB production

S. No.	Temperature (°C)	PHB (µg/ ml)
1	25	157
2	30	204
3	37	284
4	42	232

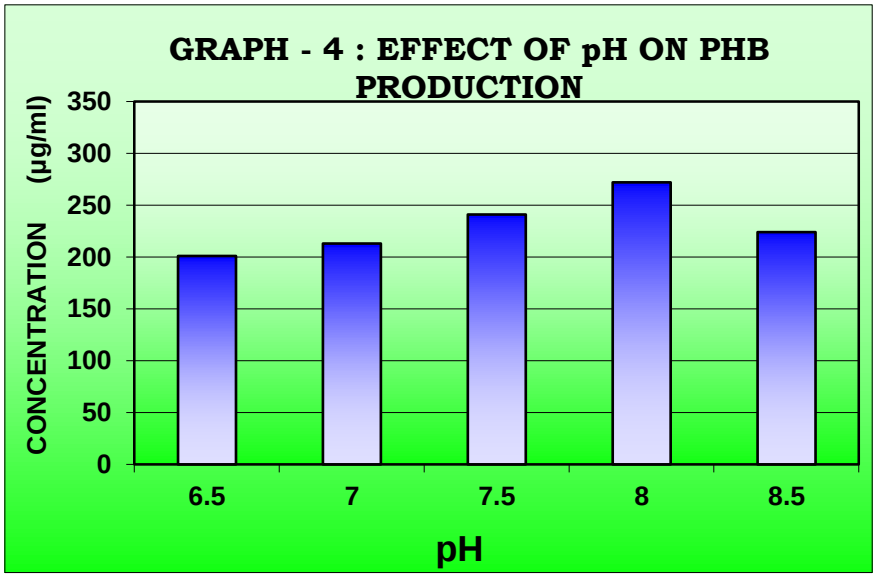


Effect of pH

The optimal pH for PHB was determined by analyzing the PHB production using phenol – sulphuric acid. Based on the readings it was observed that the selected strain shows maximum PHB production when it was maintained at pH 8. (Table 4 & Graph 4).

Table - 4: Effect of pH on PHB Production

S .No.	pH	PHB (µg/ ml)
1	6.5	201
2	7	213
3	7.5	241
4	8	272
5	8.5	224

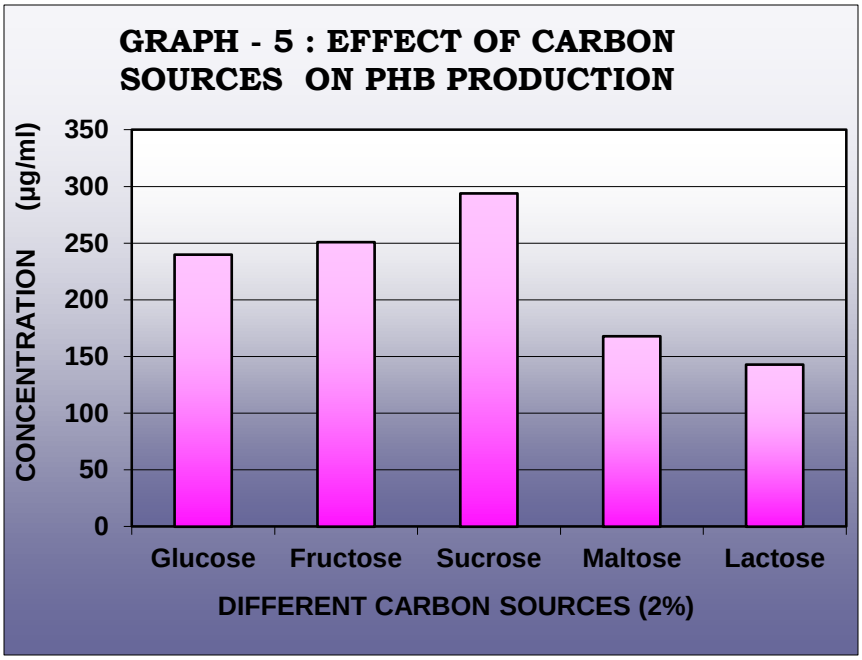


Effect of Carbon Sources

Different carbon sources were screened for maximum production of Polyhydroxybutyrate for the selected isolates (MRS medium). As it is seen from Table- 5, except for maltose, the rest of the carbon sources gave satisfactory production of PHB. However if maximum productivity was considered Glucose was taken as best carbon source. (Table 5 & Graph 5).

Table – 5: Effect of Different Carbon Sources on PHB Production

S. No.	Carbon source	PHB (µg/ ml)
1	Glucose	240
2	Fructose	251
4	Sucrose	294
5	Maltose	168
6	Lactose	143

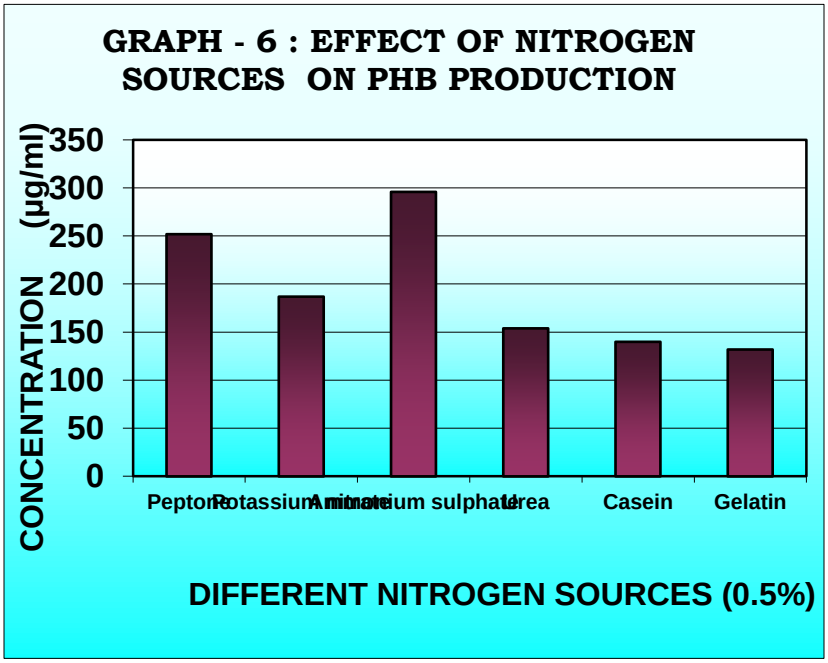


Effect of Nitrogen Sources

The nitrogen sources are of secondary energy sources for the organisms which play an important role in the growth of the organism and the production. Different nitrogen sources were screened for maximum production of Polyhydroxybutyrate for the selected isolates. As it is seen from Table-6, the lowest Polyhydroxybutyrate production was obtained with casein and gelatin. Peptone and Potassium nitrate gave much more satisfactory result among the selected nitrogenous source. Since productivity is concerned, Peptone shows the maximum PHB production and considered as best sources in this study. (Table 6 & Graph 6).

Table – 6: Effect of Different Nitrogen Sources On PHB Production

S. No.	Nitrogen sources	PHB (µg/ ml)
1	Peptone	252
2	Potassium nitrate	187
3	Ammonium sulphate	296
4	Urea	154
5	Casein	140
6	Gelatin	132

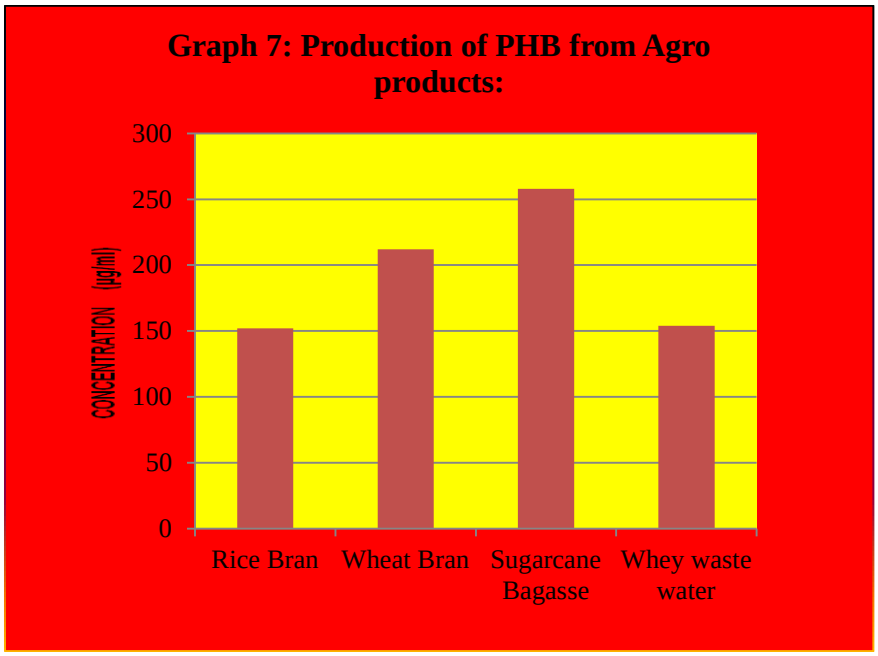


Production of PHB from Agro products:

The most economical and valuable bioproducts are produced from the natural sources and industrial wastes. One of the limiting factors in the commercial success of PHB and other PHAs production schemes is the cost of the sugar substrate used for PHA formation. In this study, several natural and agro products have been used as substrates. The results revealed that, maximum production was observed in sugar cane bagasse (Table 7 & Graph 7).

Table – 7: Production of PHB from Agro products

S. No.	Cheap sources	PHB (µg/ ml)
1.	Rice Bran	152
2.	Wheat Bran	212
3.	**Sugarcane Bagasse**	**258**
4.	Whey waste water	154



DNA ISOLATION

The genomic DNA was isolated from the selected isolate. The sample was run in 0.7% agarose gel containing ethidium bromide and the band was observed under UV- transilluminator confirming the presence of genomic DNA.

DISCUSSION

Polyhydroxybutyrate (PHB) produced by many bacteria has been investigated by microbiologists, molecular biologists, biochemists, chemical engineers, chemists, polymer experts, and medical researchers over the past many years. Applications of PHB as bioplastics, fine chemicals, implant biomaterials, medicines, and biofuels have been developed. Companies have been established or involved in PHB-related R&D as well as large-scale production. PHB synthesis has been found to improve the robustness of non-PHB-producing microorganisms and to regulate bacterial metabolism, leading to yield improvement for some bacterial fermentation products.

In this study, the selective isolate among the PHB positive isolates was subjected for the analysis different incubation time for the production of PHB. The isolate analyzed between 0 hour and 72 hours in Nutrient Broth medium (Table 2). It was determined that PHB production was gradually increased and the maximum production was observed between 36th hour and 66thhour. Maximum production of PHB was observed during 48th hour of incubation. After 66th hour of incubation, PHB production was gradually decreased.

Contradictory effects of incubation time on PHB production by various microorganisms have been reported. Nur *et al.,* (2004) in their investigation found that a best PHB production was observed after 45th hour. Sangkharak, K. and Prasertsan, P (2008), indicated that PHB was a growth associated product and its accumulation significantly increased when all cultures reached the exponential phase (after 18 hrs) till stationary phase (about 48-60 hrs). The maximum values were achieved at 60 hrs cultivation. Ram

Kumar Pandian *et al.,* (2009), assess the effect of time on the production of PHB and reported that *Brevibacterium casei* SRKP2 shows the maximum PHB production (0.135 g/L) at 48 hours.

The effect of temperature is highly variable and is dependent on the strain used and the experimental conditions. The influence of temperature on bacterial growth and PHB production is presented in Table 3. Four temperatures were tested: 25, 30, 37 and 42°C. In all strains, there was a direct relationship between PHB production, growth and temperature. Maximum PHB production was attained at 37°C.

In order to improve PHB production of the selected isolate, the influence of pH was studied. Results of PHB production are shown in Table 4. pH affects both growth and PHB production. There was a general increase in PHB production and growth of bacteria with increasing pH. Maximum PHB production was observed at a pH of 8 in the production medium when compared with other setup.

Various investigators have involved their efforts in examining the effects of pH on PHB production.

Ram Kumar Pandian *et al.,* (2009), reported the optimum growth was observed at pH 8.0.Change in initial pH of the medium showed a strong influence on the production of PHB. Even a slight difference in pH from the optimum point denoted a sudden reduction in PHB production. Initial pH value of 7.5, gave the maximum production of PHB of 25%. (Ramadas *et al.,* 2009). However, the optimal pH for PHB production is often close to 7.5 (Vishnuvardhan Reddy *et al.,* 2009) which is in agreement with our findings.

Carbon source in the culture medium has been found to affect the yield and composition of PHB production. Different carbon sources (Table- 5) were used for the optimization studies in the production medium. As it is seen from Table 5, Sucrose, besides glucose and fructose was found to be more suitable for PHB accumulation.

The biomass and biodegradable polymers were produced by QGR when fructose used as the carbon sources and yeast extract used as a nitrogen sources, respectively. Anderson and Dawes (1990), Braunegg *et al.,* (1978) showed accumulation of PHB by *Alcaligenes faecalis* using fructose as the carbon source. Sucrose, besides glucose and fructose was found to be more suitable for cell growth as well as PHB accumulation (69.4 % dry cell weight) by *Bacillus mycoides* RLj B-017 and accumulation of PHB was observed to be growth associated. Similar results were reported for *Rhizobium meliloti* by Kshama *et al.,* (2004).When sucrose was used at optimum level (55 g l–1), maximum quantities of biomass and PHB yields were found at highest concentration of CSL (25 g l^{-1}). The PHA production using different fermentable sugars was tested and it was found thatmaximum biomass was produced with 2% (w/v) sucrose (Praksah, 2008).

Different nitrogen sources were screened for maximum production of polyhydroxybutyrate for the selected isolate. As it is seen from Table - 6, among the Peptone, Potassium nitrate, Ammonium sulphate, Urea, Casein, Gelatin used for polyhydroxybutyrate production studies, Ammonium sulphate shows a higher productivity rate.

The highest value of PHB concentration (gl-1) for both strains was attained in different media supplemented with ammonium sulfate after 96 hours incubation. On the other hand, the best growth was observed with ammonium salts like chloride, sulphate, oxalate and phosphate. These results prove that the yield of PHAs is not related to the increase in growth. Beaulieu *et al.,* (1995) reported the production of PHB by *Alcaligenes eutrophus* in a synthetic medium with 3% glucose supplemented with several ammonium substrates and found that the best growth and PHB production were obtained with ammonium sulphate as nitrogen source. These results are in line with those obtained by Grothe *et al.,* (1999).

The major restriction in the commercialization of bioplastic is their high production cost. The use of readily available cheap agro-industrial residues as the carbon sources may reduce the higher cost. Several studies have shown the utilization of various carbon sources for different bacterial strains. In our study different agro products like rice bran, wheat bran, sugarcane bagasse and whey waste water from dairy industry utilized for PHB production. Among the cheap sources sugarcane bagasse shows the best cheap substrate for PHB production.

The effect of different concentrations of cane molasses has been studied by Beaulieu *et al.,* (1995) which showed that the production of PHB was high between 0.1–0.3 g/l. However, the percentage of PHB on the basis of cell dry weight was higher with a 0.1% concentration. Maximum production of PHB was obtained with cane molasses and glucose as sole carbon sources (40.8, 39.9 per mg cell dry matter, respectively). The best growth was obtained with 3% molasses, while maximum yield of PHB (46.2% per mg cell dry matter) was obtained with 2% molasses (Mona *et al.,* 2001) which is similar to our studies.

CONCLUSION

The ***Bacillus subtilis*** was isolated from isolated from garden soils collected in a sterile container from different parts of Chennai. The isolated strain was screened for the production of Polyhydroxybutyrate. After growth study, the production was done by shake flask fermentation. The various factors affecting production of Polyhydoxybutyrate was assayed, which include temperature, pH, different carbon and nitrogen sources, and different agro substrate. Results showed that pH 8 and temperature 37ºC is an optimum environmental parameter for the growth of the isolate and for its better production. In addition to this, sucrose was found to be better carbon source, ammonium sulphate as a better nitrogen source, sugar cane bagasse as a better agro substrate for better production of Polyhydrxoybutyrate. Based on the results of the present study, it is concluded that *Bacillus subtilis* isolated from soil samples showed better characteristic PHB producing ability.

ACKNOWLEDGMENT

I express my deep sense of gratitude and indebtedness to my Guide and Supervisor **Dr. S. NATARAJAN,** Department of Plant Biology and Plant Biotechnology, Guru Nanak College, Chennai - 600 042, for his inspiring guidance and unhesitating support throughout the period of study.

REFERENCES

1. Alistair J. Anderson and Edwin A. Dawes.(1990). Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates.*Microbiological Reviews*.54 (4): 450-472.
2. Beaulieu, Yvan Beaulieu, Joelle Melinard, Sithian Pandian, and Jacques Goulet. (1995). Influence of ammonium salts and cane molasses on growth of *Alcaligenes eutrophus* and production of polyhydroxybutyrate. *Applied and Environmental Microbiology*, 61(1): 165–169.
3. Braunegg. G, Lefebvre. G, and Genser. K. F. (1998). Polyalkanoates, BiopolyesterFrom Renewable Resources: Physiological and Engineering Aspects. *Journal of Biotechnology*, 65: 127-161.
4. Das, Q., Chohury, J.U., Anwar, M.N. 2004. Isolation, Purification and Characterization of Biodegradable Polymer Producing Bacteria Pseudomonas pseudomallei. Int. J. Agri. Biol., 7(1): 114 -117.
5. Grothe, E., M.M. Young and Y. Chisti, 1999. Fermentation optimization for the production of poly(â- hydroxybutyric acid) microbial thermoplastic. Enzyme Microb. Technol., 25: 132-141.
6. Guo-Qiang Chen.(2010). Plastics from Bacteria, Natural Functions and Applications. Springer-Verlag Berlin Heidelberg
7. Jose .M. Luengo, Bele .N. Garcıa, Angel Sandoval, German Naharroy and Elıas R Olivera. (2003). Bioplastics from microorganisms.*Current Opinion in Microbiology*, 6: 251–260.
8. Kshama *et al.,* (2004). Studies on the production of Biopolymer by Rhizobium species, their Isolation and Characterization Institutional repository of CSIR- CFTRI. 82: 6231– 6235
9. LAW, JOHN H. (Harvard University, Cambridge, Mass.) AND RALPH A. SPLEPECKY 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol.**82:**33–36.
10. Lee, S. Y. (1996). Review Bacterial Polyhydroxyalkanoates. *Biotechnology & Bioengineering*., 49:1-14.
11. MiracYilmaz, HalukSoran, and YavuzBeyatli (2005). Determination of poly-*β*-hydroxybutyrate (PHB) production by some Bacillus spp. *World Journal of Microbiology & Biotechnology*, 21: 565–566.
12. Mona K. Gouda., Azza E. Swellam, Sanaa H. Omar. (2001). Production of PHB by a *Bacillus megaterium*strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources.*Microbiological Research,*156: 201–207.
13. Nisha V. Ramadas, Sudheer Kumar Singh, Carlos Ricardo Soccoland Ashok Pandey.(2009). Polyhydroxybutyrate Production using Agro-industrial Residue as Substrate by *Bacillus sphaericus*NCIM 5149.*Brazilian Archives Of Biology And Technology,*52: 17-23.
14. NurYuksekdag.Z., BelmaAslim, YavuzBeyatli and NazimeMercan.(2004). Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis*25 and *Bacillus megaterium*12.*African Journal of Biotechnology,*3 (1): 63-66.
15. Poirier, Y., C. Nawrath and C. Somerville, 1995. Production of polyhydroxyalka-noates, a family of biodegradable plastics and elastomers, in bacteria and plants.Biotechnol., 13: 142-150.

16. Prakash M. Halami. (2008). Production of polyhydroxyalkanoate from starch by the native isolate Bacillus cereus CFR06.*World Journal of Microbiology and Biotechnology*, 24: 805–812.
17. Ram Kumar Pandian, Kalimuthu Kalishwaralal, Sangiliyandi Gurunathan. (2009). Purification, immobilization, and characterization of nattokinase on PHB nanoparticles. *Bioresource Technology* , 100 : 6644–6646.
18. Sangkharak K and Prasertsan P (2008) Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. Electronic Journal of Biotechnology 11: 83-94.
19. Sudesh kumar *et al*., (2000). Polyhydroxyalkanoates: Bio-based microbial plastics and their properties. *Malaysian Polymer Journal,* 2(2): 31-57.
20. Vishnuvardhan Reddy.S., Thirumala .M., and Mahmood.S.K. (2009). Production of PHB and P (3HB-co-3HV) biopolymers by Bacillus megaterium strain OU303A isolated from municipal sewage sludge. *World Journal of Microbiology and Biotechnology*, 25: 391–397.
21. Wu Q., Sun S.Q., Yu P.H.F., Chen A.X.Z., Chen G.Q., 2000, Environmental dependence of microbial synthesis of polyhydroxyalkanoates, Acta Polym. Sin., 6, 751–756.