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RESEARCH ARTICLE

Design, Screening and Microbial Synthesis of Bio-polymers of Poly-Hydroxy-Butyrate (PHB) from Low Cost Carbon Sources

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Abstract

The environmental pollution by petro based plastics a cause of concern, which are non biodegradable. Hence biodegradable, biologically synthesized polymers with similar properties of conventional plastic are sought. Poly-β-hydroxy-butyrate (PHB) is a member of a family of polyhydroxyalkonates synthesized by numerous bacteria as an intracellular carbon and energy storage compound under nutrient-limiting conditions with excess carbon. Among microbially formed Poly HydroxyAlkanotes (PHA) is biodegradable, water soluble, non-toxic, bio-compatible, piezoelectric, thermoplastic and elastomeric. It has wide applications in different areas such as medicines, long term dosage of drugs, cosmetic world, cosmetic containers, shampoo bottles, insecticides, fertilizers, packing materials, disposable items such as razors, diapers, feminine hygienic products etc.

Media formulations were designed with different compositions of Molases/Glycerol/Glucose for enhancement of bio-polymer synthesis. Microbes were screened for PHB production by Sudan black staining of the colonies. Cells were broken with hypochlorite and PHB extracted into chloroform and its content was analyzed by Spectrophotometer at 235nm using crotonic acid as standard. Ten isolates out of 16 could produce PHB. Isolate #10 gave 38.46% PHB when grown on bio-diesel derived glycerol whereas isolate # 3 gave only 8% PHB when grown on molasses.

INTRODUCTION

The environmental negative impact caused by the disposal of plastics as well as the progress of biotechnology has motivated the research on biodegradable and biocompatible polymers [Lee. S.Y. Et al., 1999]. Amongst all, microbially formed polyhydroxyalkanoates (PHA) offer much more potential for significant contribution as bio plastic.

PHAs are a class of natural polyesters which can be produced and accumulated by many Gram- positive and Gram-negative bacteria. Polyhydroxybutyrate (PHB) is the most wide spread and thoroughly characterised PHA found in bacteria. These polymers are accumulated intracellularly under conditions of nutrient stress and act as a carbon and energy reserve [Senior.P.J, Et al., 1998]. The characteristic of this polymer is similar to synthetic plastic or petrochemical-based plastics. .PHAs is biodegradable, water insoluble, non toxic, bio-compatible, piezoelectric, thermoplastic, and elastomeric [Brandl. H., Et al., 2000]. It has wide application in different areas such as

packaging material, long term dosage of drugs, medicines, insecticides, fertilizers, cosmetic world, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetics containers, shampoo bottles, cups etc.

Due to the high production cost of PHAs, the use of PHAs as substitutes for the conventional synthetic polymeric material with a wide range of applications has been compared. The use of cheap carbon sources and nutritional supplements with feeding substrate strategies are required in order to reduce the production cost of biopolymer [Kim.S. Lee. Et al., 2012]. For the production of PHB, molasses originated from sugar cane and glycerol generated during biodiesel production have been utilised as carbon sources. The use of these low cost carbon sources can downsize the production costs and this improves the market competitiveness through considerable cost savings.

The purpose of the present research study was to synthesize PHB bio-polymer with cheap carbon sources locally available.

Materials and methods

Collection of samples

Soil sample, sewage sludge and leaves from Weed Plants were collected from RVCE, Bangalore (India) Campus and used for isolation of bacteria.

Isolation of bacteria from different samples

Various samples collected were serially diluted and plated on nutrient agar medium plates. The plates were incubated at 37^0 C for 24-48 hrs isolated colonies were sub cultured till the single colony was obtained and preserved on nutrient agar slant at 4^0 C.

Screening for PHB producing bacteria

All the bacterial isolates were qualitatively tested for PHB production using Sudan Black B dye. For rapid screening of PHB producers, nutrient agar medium was autoclaved and poured into petri plates and allowed for solidification. The plates was divided into 8 equal parts and in each part a bacterial isolate was inoculated. The plates were incubated at 30^0C for 24-48 hrs. Ethanolic solution of (0.02%) of Sudan black was spread over the colonies and the plates were kept undisturbed for 30 minutes [Hartman 1940]. They were then washed with 98% ethanol to remove the excess stain from the colonies. The dark blue colored colonies were taken as positive for PHB production.

Production media

DSMZ media (Deutsche Sammlung von Mikroorganismen und Zellkulteren)

carbon source- 2%(Molasses / Glycerol), NH_4Cl- 0.5g/L, KH_2PO_4- 2.3g/L, Na_2HPO_4- 2.3g/L $MgS0_4.\ 7H_2O$- 0.5g/L, $CaCl_2$- 0.01g/L, Ferrous sulphate- 0.5g/L, Yeast extract-0.16g/L. 100ml media was prepared, autoclaved and used for PHB production by the organisms.

Experimental conditions

Batch experiments were performed in shake flasks for production of the biopolymer. Organisms were inoculated and incubated for 48 hrs at 30^oC, 120 rpm, and pH 7.

After 48 hrs they were transferred to nitrogen deficient media and incubated for 48 hrs. Then the PHB produced was extracted using chloroform method and quantified using crotonic acid standard graph.

Extraction and quantitative analysis of PHB

Sample was taken from the production media. Centrifuged at 10,000 rpm at 4˚c for 20min. The supernatant was discarded and 0.1ml of acetone was added to the pellet and keep till it dries. After the acetone is dried 1:1 of 500µl of NaClO and chloroform was added. (NaClO cleaves the cell wall and chloroform dissolves PHB) Mixture was mixed thoroughly and kept for about 30min. Then centrifuged at 10,000 rpm for 30min at 4˚c for 20min. Three layers were formed: top NaClO, middle cell debris and bottom chloroform with PHB. The chloroform layer was pipetted into glass bottles. Kept out till the chloroform dries. Then 5ml of sulfuric acid was added into the bottles and kept in hot air oven at 100˚c for 1hr which converts it to Crotonic acid (add extra sulfuric acid if reading is high) The absorbance was taken at 235nm using sulfuric acid as blank in UV Spectrophotometer [John and Ralph, 2001].

Quantification of PHB by Spectrophotometry

Crotonic acid powder was dissolved into sulfuric acid and standard solution of 0.1µg of Crotonic acid/ µl of sulfuric acid was prepared. Working STDof 5, 10, 15, 20, 25, 30, 40, 50µg/3m of sulfuric acid were prepared. Blank was prepared by adding 3 ml of sulfuric acid. Took the absorbance reading at 235nm. Standard graph of concentration v/s absorbance was prepared [Law and Slepecky, 1999].

Results and discussion

Isolation of representative bacteria from various samples

We isolated 16 bacteria from soil, air, sewage sludge and Plant leaf surface. Of these 8 isolates were proved to be positive for PHB production (using Sudan black staining in the plate).

Selection of promising isolates

The organisms which are stained dark, those microbes were selected and screened for producing PHB using glucose as standard carbon source. Also they were cultivated in the media containing molasses and glycerol as carbon sources.

Based on the colony staining, 8 promising isolates were selected covering all the sources of the isolates, which include isolate 2, 3, 6, 9, 10, 11, 14, 16, and Bacillus megaterium. The microbial isolates, PHB Concentration and Percentage of Yield was Shown in Table and Graphical Figures- 1.2.3 and 4 respectively.

Quantification of PHB by Spectrophotometry

Sl. No.	Crotonic acid µl	Sulphuric acid ml	Concentration µg	O.D reading at 235nm
1	-	3	-	-
2	50	2.950	5	0.085
3	100	2.900	10	0.132
4	150	2.850	15	0.135
5	200	2.800	20	0.301
6	250	2.750	25	0.477
7	300	2.700	30	0.512
8	400	2.600	40	0.727
9	500	2.500	50	0.778

Table1: Absorbance of Crotonic acid at 235 nm:

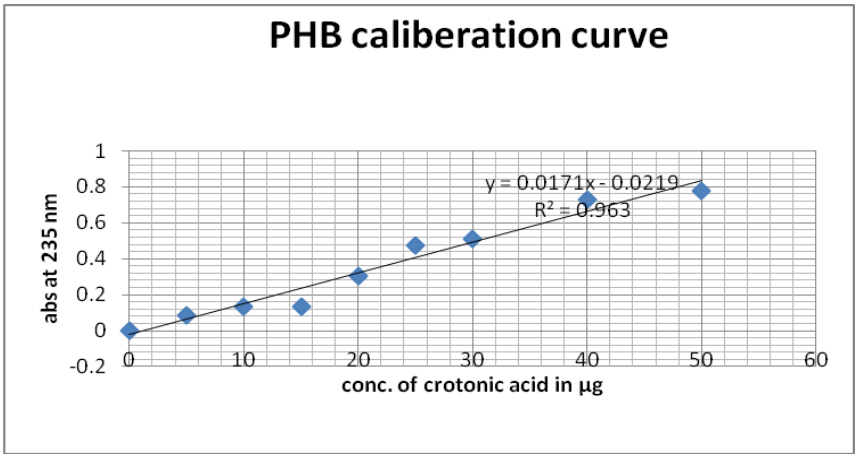

Fig 1: Standard graph of Crotonic acid for quantifying PHB

Microbial Isolates	PHB Concentration in g/100ml	PHB yield in %
Isolate 2	0.0063	11.6
Isolate 3	0.0123	12.05
Isolate 6	0.015	26.3
Isolate 9	0.0018	1.76
Isolate 10	0.0007	7.0
Isolate 11	0.0015	2.3
Isolate 14	0.0006	5.45
Isolate 16	0.0003	1.42
Bacillus	0.012	14.11

megaterium		

Table 2 : Effect of glucose on PHB production.

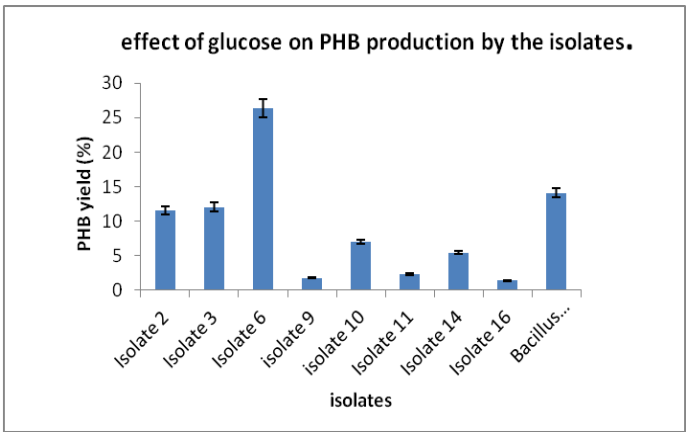

Fig 2: Graph representing the effect of glucose on PHB production by the selected isolates.

The data in the table depicts the effect of glucose on the growth and production of PHB by the selected isolates. Amongst the different PHB isolates, isolate 6 was significantly superior when compared to all other isolates. Although isolate 3 had good growth in the glucose media, it can be seen that it is less efficient in producing the PHB.TheBacillus megaeriumwas the next best. The rest of the other isolates were found to be ineffective.

Microbial Isolates	**PHB Concentration in g/100ml**	**PHB yield in %**
Isolate 2	0.0117	4.17
Isolate 3	0.005	2.3
Isolate 6	0.0114	3.23
Isolate 9	0.0117	1.03
Isolate 10	0.008	3.52
Isolate 11	0.0016	0.93
Isolate 14	0.0007	7.5
Isolate 16	0.005	2.80
Bacillus megaerium	0.0123	6.75

Table 3: Effect of molasses on PHB production.

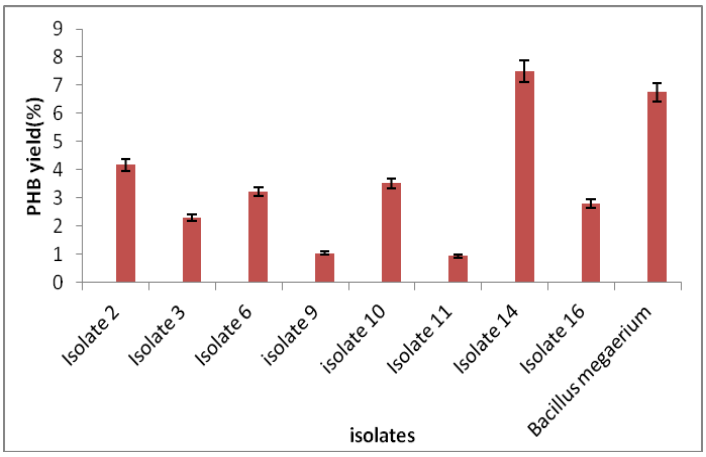

Fig 3: Graph representing the effect of molasses on PHB production by the selected isolates.

The data in the table depicts the effect of molasses on the growth and production of PHB by the selected isolates. Amongst the different PHB isolates, isolate 14 was significantly superior when compared to all other isolates. Although isolate 2 and 3 had good growth in the molasses media, it can be seen that they are less efficient

in producing the PHB.TheBacillus megateriumwas the next best. The rest of the other isolates were found to be ineffective.

Microbial Isolates	PHB Concentration in g/100ml	PHB yield in %
Isolate 2	0.011	20.8
Isolate 3	0.0112	29.47
Isolate 6	0.005	9.75
Isolate 9	0.015	7.69
Isolate 10	0.005	38.46
Isolate 11	0.0009	3.9
Isolate 14	0.0018	3.0
Isolate 16	0.004	25
Bacillus megaerium	0.005	4.82

Table 4: Effect of glycerol on PHB production.

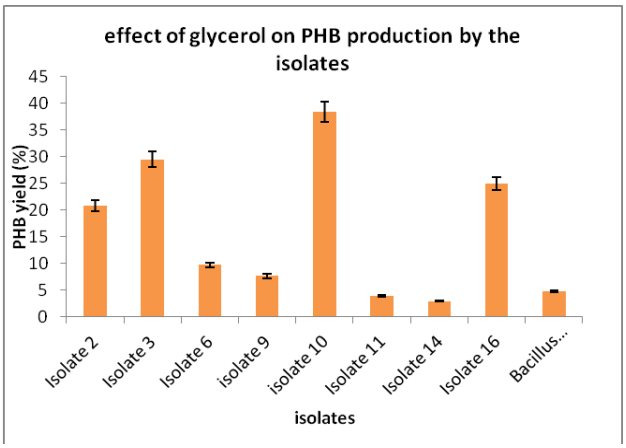

Fig 4: graph representing the effect of glucose on PHB production by the selected isolates.

The data in the table depicts the effect of glycerol on the growth and production of PHB by the selected isolates. Amongst the different PHB isolates, isolate 2a, with the PHB accumulation of 0.005g/100ml, was significantly superior when compared to all other isolates. Although isolate 2 and 3 had good growth in the molasses media, it can be seen that they are less efficient in producing the PHB. The isolate 3 and 16 were the next best isolates. The rest of the other isolates were found to be ineffective.

Conclusion

The aim of this study was to screen and select suitable organisms for PHB production. Among the 16 isolated bacteria, isolate 10 was found to be more efficient for PHB production in the media containing glycerol as the carbon source with PHB content of 38.46%, and DCW 0.013g/100 ml. isolate 3 was also efficient with PHB content of 29% of its DCW.

Although molasses proved to be good carbon source for the growth of the bacteria, these isolates were less efficient in making use of it for PHB production. Isolate 6 gave PHB content up to 26% of its DCW, in the media with glucose as the carbon source.

Non-solvent based extraction techniques for extraction of PHB needs to be developed, as it will help to significantly reduce PHB production cost. Also the cultural parameters have to be optimized with other stress conditions so that the biomass and in turn PHB yields can be increased. .

The focus of future research would be to reduce the cost of production as well as improve the quality of the polymers to make it suitable for high cost products. A balance between operating cost, product yield and quality is necessary to make it more economical.

References

1. A.P. Bonartsev, V.L,Myshkina, D.A.Nikolaeva, E.K.Furina, T.A.Makhina, V.A.Livshits, "Biosynthesis, biodegradation and application of PHB and its copolymers- natural polyesters produced by diazotrophic bacteria", Communicating Current research and Educational topics and Trends in Applied Microbiology, 2007, pp 295-307.
2. N, Ramana. K. V "Identification and Characterization of PHB producing Bacillus cereus and Bacillus mycoides strains", International Journal of Environmental Sciences, Vol 1(5), 2011, pp 744-756.
3. Chenyu Du, Julia Sabirova, WimSoetaert, Sze Ki Carol Lin, Polyhydroxyalkanoates Production From Low-cost Sustainable Raw Materials, Current Chemical Biology, 2012, Vol 6, pp 14-25
4. Edwin a. Dawes, "Polyhydroxybutyrate: an Intriguing Biopolymer", Bioscience Reports, Vol. 8, No. 6, 1988, pp 537-547.
5. F. Karbasi, M. Ardjmand, H. Yunesi, A. Safe Kordi, S. Yaghmaei, "Investigation of Optimum Fermentation Condition for PHA Production by four species: Hydrogenophagapseudoflava DSMZ 1034, Azohydromonaslata DSMZ 1123, Cupriavidusnecator DSMZ 545 and Azotobacterbeijinckii DSMZ 1041", World Applied Sciences Journal 14, 2011, 36-47
6. K. Van de Velde, P. Kiekens, "Biopolymers: overview of several properties and consequences on their applications", Elsevier, Sept 2001, pp 433-442.
7. KanokphomSangkharak, PoonsukPrasertsan, "Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition", Electronic journal of Biotechnology, 2008 Vol 11 page no 1-12.
8. M. Sarifzadeh, G.D. Najafpur, H. Younesi, H. Eisazadeh, " PHB Synthesis by Cupriavidusnecator DSMZ %$% Utilising various Carbon sources", World Applied Sciences Journal, 2009, Vol 7(2), 157-161
9. Nazia Jamil, Nuzhat Ahmed, "Production by Pseudomonas aeruginosa isolated from Marine Source", Brazilian Archieves of Biology and Technology, June 2208, Vol 51, 457-464.
10. Paramjit Singh, Nikita Parmar, "Isolation and Characterisation of two novel PHB producing bacteris", African Journal of Biotechnology, June 2011, vol 10(24) 4907-4919
11. Parth N. Patel, Khushboo G. Parmar, Alpesh N Nakum, mitul N Patel, Vanita R patel, Dr. DhruboJyoti Sen, " Biodegradable polymers: An Ecofriendly Approach In Newer Millenium", Asian journal of Biomedical and Pharmaceutical sciences, 2011, Vol1(3),23-29
12. Pin-Yuan Tian, Longan Shang, Hong Ren, Yu Mi, Dai-Di Fan and Min Jiang, " Biosynthesis of Polyhydroxyalkanoates: Current research and development", African Journal of Biotechnology, March 2009, Vol 8(5) , page no 709-714.
13. RamchanderMergu, A. Sridhar Rao, D. Ramesh, S. Girisham, S.M. Reddy, "Production of PHB under Aerobiic dark conditions by two anoxygenic phototrophic purple non sulphur bacteria isolated from tannery effluent", International Journal of Chemtech Research", Sept 2012, Vol 4, 1103-1107
14. SrividyaShivakumar, "Accumulation of PHB by Microbacteriumbarkeri DSM 20145", Turk J Biol, 2012, Vol 36, 225-232.
15. Zachary T. Dobroth, Shengiun Hu, Erik R. Coats, Armando G. McDonald, "PHB synthesis on biodiesel wastewater using mixed microbial consortia, Bioresource Technology, 102, 2011, 3352-3359.