International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.11 pp 224-228, 2016

Isolation and Identification of Polyhydroxybutyrate(PHB) Producing *Bacillus cereus* BB613-A Novel Isolate.

B. Bharathi, D. Gowdhaman* and V. Ponnusami

Bioprocess Intensification Lab, School of chemical & Biotechnology, SASTRA University, Thanjavur, India

Abstract : Poly-3-hydroxy butyrate (PHB) is a copolymer of polyhydroxyalkonate family. It accumulates in the intracellular granules of bacteria under nitrogen limiting environment. The PHB producing bacteria was isolated from farm soil by serial dilution method. The Sudan Black B staining and Nile Blue A staining were carried out to confirm that the isolated strain was capable of producing PHB. Further, it was subjected to morphological, biochemical and molecular characterization. The 16S rRNA sequencing and phylogenetic analysis confirmed the organism was *Bacillus cereus* BB613 with accession number LN613102. The growth parameter and usage of low cost substrate for production was optimized. The maximum PHB accumulation was found at 30°C and pH 7.0 after 48h incubation. For the production of PHB, 1% sucrose was used as carbon source. Sucrose was then substituted by low cost substrates such as mosambi peel, orange peel, banana peel and molasses which gave yields 35.2%, 26.7%, 32.1% and 33.9% respectively.
Key words: Poly-3-hydroxybutyrate, 16Sr RNA sequencing, Phylogenetic analysis, Mosambi peel.

1. Introduction

Synthetic thermoplastics is a type of solid waste pollutant which affects the environment and cannot be degraded [1]. To overcome this problem is to producing biodegradable polymer. Poly-3-hydroxy butyrate (PHB) is a copolymer of polyhydroxyalkonate family. It accumulates in the intracellular granules of bacteria in the excess of carbon and less nitrogen. Poly-3-hydroxy butyrate is ecological polymer, reusable, insoluble in water and non-toxic [2]. The nature of the material cause suitable for the application of packaging [3]. Many of the bacterial species has the ability to produce PHB, they are *Bacillus megaterium, Bacillus thuringiensis, Halomonas campisalis, Brevibacterium casei, Pseudomonas sp,* etc[4-10] . It has a wide application in various fields like drug delivery vehicles in medicine [11-13], plant germination and plant growth in agriculture [14], Nano fibrous scaffolds are used for cell proliferation, cell signaling in tissue engineering [15-18]. Though there are many reports to minimize the production cost of PHB by agro industrial residues [19-25] like palm oil, molasses, rice bran, wheat bran, and soya bean etc.,. The main aim of the work was to produce PHB from isolated bacteria and also to improve the production of PHB by using economically low cost carbon sources like mosambi peel, orange peel, banana peel, molasses.

2. Materials and Method

2.1 Isolation of Bacteria from Soil

The bacteria were isolated from farm soil, because of the presence of partially degraded plant waste and usage of fertilizers there will be rich in carbon and less nitrogen source. The bacteria was isolated by serial dilution method and inoculated onto the nutrient agar plate. The Bacterial colony was preserved in a slant at 4°C for further characterization.

2.2 Screening of PHB Producer

The bacteria which produce PHB granules were screened by Sudan black B and Nile blue A staining. 0.3% of Sudan black B in 70% of ethanol was prepared. Heat fixed smear was stained with Sudan black B solution and incubated for 10minutes. Then rinsed with tap water, counter stained with safranin for 5minutes, visualized under phase contrast microscope at 1000X magnification. For Nile blue staining, 0.1% of Nile blue A solution was added into the heat fixed smear followed by incubation at 55°C for 10 minutes. After incubation rinsed with tap water to remove excess stain and observed under fluorescent microscope.

2.3 Biochemical and Molecular Characterization

The isolated bacterial strain was described by Biochemical Test as per Bergey's Manual of Determinative Bacteriology and 16s rRNA sequencing, in an ABI 3730xl sequencer (Applied Bio systems). Phylogenetic studies was done for the conformation of the bacteria using Phylogene.fr. The sequence was submitted in webin to obtain the accession number.

2.4 Production of PHB

Bacillus cereus BB613 (1ml) was inoculated in the media containing (Yeast extract 2.0g/l), Peptone (2.0g/l), K_2HPO4 (2.0g/l), $(NH4)_2SO_4$(2.0g/l), Mgso4(0.3g/l), Sucrose (10g/l) for the production of PHB and it was incubated at 37°C for 48 hrs at 150rpm. Every 12 h, samples were with drawn and it was subjected to centrifugation at 10,000rpm for 10 min and the cell pellet was collected.

2.5 Optimization of Process Parameters for PHB Production

The optimization of various process parameters like temperature (20, 30, 40, 50°C), pH (5.0, 6.0, 7.0, 8.0and 9.0), time duration (12, 24, 36, 48h), carbon source (1%) like orange peel, banana peel, mosambi peel, molasses were used. 1ml of inoculum was added into the medium and PHB production was determined.

2.6 Extraction of PHB

After 48h of incubation, samples were with drawn and it was then subjected to centrifugation at 10000 rpm for 10 minutes. The cell pellet was dried at 60°C in a hot air oven and cell dry weight was measured. Then 3ml of 6% sodium hypochlorite was added and incubated at 50°C for 20minutes and 3ml of hot chloroform was added into the pellet. From the three phases, chloroform containing PHB was carefully collected and precipitated by 5ml methanol or hexane. The PHB content was determined by spectrophotometric assay as described by Law and Slepecky (1960) .

3 Results & Discussions

3.1 Screening of PHB Producers

Sudan black B staining revealed the presence of PHB granules in the bacterial cytoplasm visualized using Phase contrast microscope 1000X magnification (Fig.1A) The PHB emits the fluorescence when stained with Nile blue A Stain visualized under fluorescent microscope range at 390nm. (Fig: 1B)

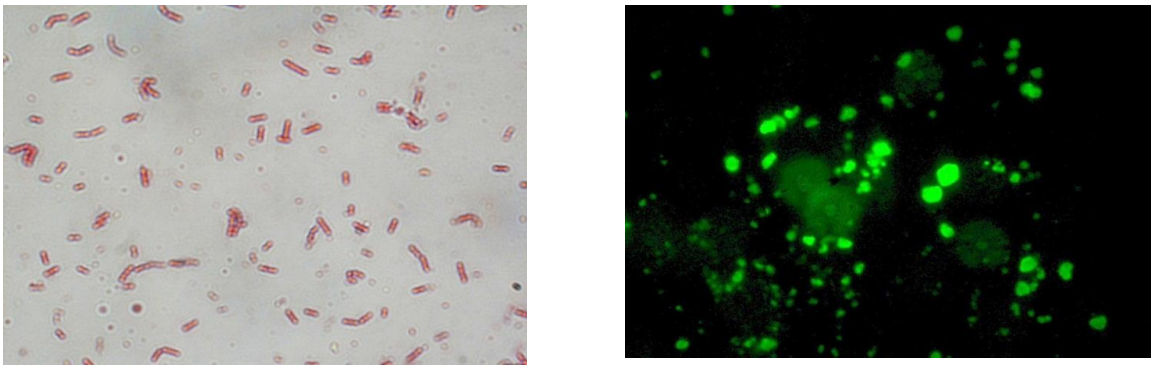

Figure 1: A) Micrograph of strain *Bacillus Cereus* BB613 with Sudan black B showing PHB accumulation when counter stained with safranin using Phase contrast microscope B) Nile blue A stain fluorescent micrograph of *Bacillus Cereus* BB613 showing green fluorescence due to PHB granules.

3.2 Strain Identification

The PHB producing positive isolate was identified as *Bacillus cereus* BB613, by the biochemical and molecular characterization analysis. Table 1 shows the biochemical characterization of isolated bacteria. The nucleotide sequence was subjected to Blast and *Bacillus cereus* BB613 was found to have 99% homology with *Bacillus cereus.*

Table 1: Morphology and biochemical characterization of *Bacillus cereus* BB613

Test	Observation
Gram's stain	Gram positive
Spore staining	Positive
Cell shape	Rod
Colony character	White, raised, irregular
Motility	Positive
Starch hydrolysis	Positive
Catalase	Positive
Indole	Negative
Methyl Red	Negative
Voges-Proskauer	Negative
Citrate Utilization	Positive
Oxidase	Positive
Urea	Positive
TSI	Acid slant/Acid butt, no gas, no H2S
Glycerol	Positive
Arginine	Positive
Mannitol	Positive
Glucose	Positive
Sucrose	Positive
Lactose	Positive

The unrooted phylogram was constructed using phylogene.fr. (Fig.2) The bacterial sequence was submitted at ENA Webin and the accession number was obtained as LN613102.

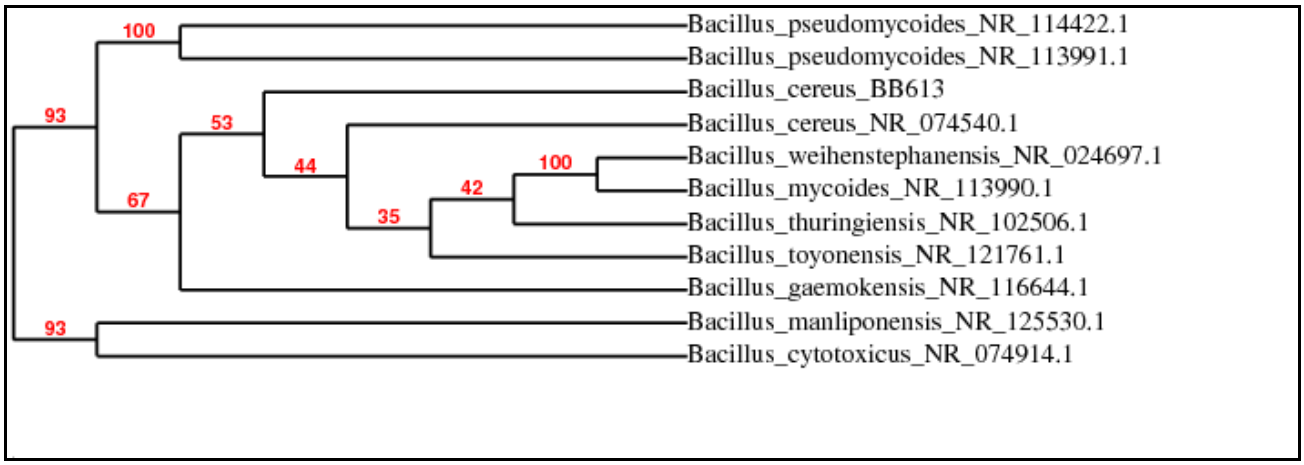

Figure 2: Unrooted phylogram obtained by neighbor-joining of 16SrRNA sequence of the isolated *Bacillus Cereus* BB613 strain.

3.3 Optimization of Process Parameter for PHB Production

The effect of process parameters on the production of PHB by *Bacillus cereus BB613* was assessed. Table 2 shows the maximum yield of the PHB produced by the *Bacillus cereus BB613* was optimized and found to be maximum of 0.534 (g/l) PHB production occurred at 48 h time interval which is the result of consumption

of PHB by cells as carbon reserve when depletion occurs. The optimum growth and production of PHB was found to be 30°C yielding 0.383g/l. The increase in growth cause decrease in production due to disruption of cell wall and also due to alternation of metabolic activity. Increase in the temperature causes decrease in the production this is due to the disruption of cytoplasmic membrane. Therefore the cytoplasm content diffuses out and PHB cannot exist from the cell (Tripathi *et al*., 2012).The maximum PHB production was obtained at pH value of 7.0 with yield of 1.765 (g/l). Substrate sucrose was replaced by different carbon source and maximum yield of the PHB production was found on Mosambi peel yielding 0.352 (g/l).

Table: 2 Yield of PHB (g/l) was produced by *Bacillus cereus BB613* at different time interval, pH, temperature and carbon source 1% (w/v)

Parameters	Biomass (g/100ml)	PHB (g/100ml)	Yield %
Time(h)			
24	**0.2113**	**0.409**	**40.96**
48	**0.3306**	**0.534**	**53.43**
72	**0.2603**	**0.203**	**20.30**
96	**0.1097**	**0.158**	**15.81**
p H			
5	**0.1214**	**0.379**	**37.91**
6	**0.2312**	**0.603**	**60.14**
7	**0.3688**	**0.621**	**62.58**
8	**0.1312**	**0.236**	**23.63**
9	**0.0267**	**0.153**	**15.39**
Temperature (°C)			
20	**0.0128**	**0.088**	**8.8**
30	**0.3332**	**0.383**	**38.8**
40	**0.1623.**	**0.248**	**24.8**
50	**0.0161**	**0.096**	**9.61**
Carbon source used			
Banana peel extract	**0.8214**	**0.312**	**32.1**
Orange peel extract	**0.8844**	**0.267**	**26.7**
Mosambi peel extract	**0.9787**	**0.352**	**35.2**
Molasses	**0.8486**	**0.339**	**33.9**

4. Conclusion

Isolated novel bacterial stain was capable of producing PHB was confirmed by characteristics staining techniques. Based on the morphological, biochemical and molecular characterization of the bacteria was identified as *Bacillus cereus BB613* and accession number was obtained as LN613102.The optimum growth and maximum PHB accumulation was found to be at temperature 37°C, pH 7.0, incubation time 48h. The maximum yield of the PHB 35.2% from 1% mosambi peel extract. From the above studies, it was concluded that the *Bacillus cereus BB613* has a capability of producing PHB, from various low cost substrates.

5. References

1. Raghul S S. Sarita G B. and Muthusamy C., Biocompatible polyhydroxybutyrate (PHB) production by marine *Vibrio azureus* BTKB33 under submerged fermentation, Ann Microbiol., 2015, 65, 455–465.
2. Hankermeyer C R and Tjeerdema RS. Polyhydroxybutyrate: plastic made and degraded by microorganisms, Rev Environ Contam Toxicol., 1999,159, 1-24.
3. Nisha V. R. Sudheer K S. Carlos R S. and Ashok P., Polyhydroxybutyrate Production using Agro-industrial Residue as Substrate by *Bacillus sphaericus* NCIM 5149 Braz. arch. biol. technol.,2009, 52,
4. Edwin A. D., Polyhydroxybutyrate: an Intriguing Biopolymer, Biosci. Reports, 1988, 8.
5. George S K , Anuj N L , Sethu P , Kumar A , Lucia E C S , Mariadhas V A , Ki C C , Joseph S and Naif A A D., Antiadhesive activity of poly-hydroxy butyratebiopolymer from a marine *Brevibacterium casei* MSI04 against shrimp pathogenic vibrios, Micr Cell Fact 2014, 13:114
6. Ayub ND, Pettinari MJ, Ruiz JA, and López NI. A., polyhydroxybutyrate-producing Pseudomonas sp. isolated from Antarctic environments with high stress resistance, Curr. Microbio., 2004, 49,

7. Chi-Ling T, Hui-J C, and Gwo-Chyuan S, Identification and Characterization of the *Bacillus thuringiensis phaZ* Gene, Encoding New Intracellular Poly-3-Hydroxybutyrate Depolymerase, J Bacteriol, 2006,5, 7592–7599.
8. Kulkarni SO, Kanekar PP, Nilegaonkar SS, Sarnaik SS, Jog JP. Production and characterization of a biodegradable poly (hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV) copolymer by moderately haloalkalitolerant Halomonas campisalis MCM B-1027 isolated from Lonar Lake, India. Bioresour Technol. 2010 Dec;101(24):9765-71.
9. Charen,T , Vaishali.P, Kaushalya,M Amutha,K Ponnusami.V and Gowdhaman.D, Isolation and identification of Polyhydroxybutyrate producing bacterial strain Bacillus thuringiensis GVP from chlorine contaminated soil, International Journal of ChemTech Research, 2014, 6, 3197-3202.
10. Mona K. G, Azza E. S, Sanaa H. O., Production of PHB by a Bacillus megaterium strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources Microbiol. Res, 2001, 156, 201–207.
11. Christopher J. B, and Anthony J. S., Applications of Polyhydroxyalkanoates in the Medical Industry, Inter. J. Biotechnol. Wellness Indust, 2012, 1 , 53-60.
12. Cesare E, Cristina B , Federica C ,and Emo C I., Poly(hydroxyalkano ates)-Based Polymeric Nanoparticles for Drug Delivery, J. Biomedi. Biotechnol., 2009, http://dx.doi.org/10.1155/2009/571702
13. Michał M , Adam A. M , Jan Z , Michał K , Piotr K., Synthesis of PHB-based carrier for drug delivery systems with pH-controlled release, Eur. Poly. Journal., 2013, 49, 4149–4156
14. D.Latha,Sandya,shahina., Biopolymer production using *Pseudomonas.sp* (MTCC) and its Application in agriculture ,Americ. J.Biochem.Molecular Biol., 2013,3,293-303.
15. Ya-Wu W, Qiong W, Guo-Q C., Attachment, proliferation and differentiation of osteoblasts on random biopolyester poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) scaffolds, Biomaterials, 2004, 25, 669 – 675.
16. Purushothaman. K, Swaminathan. S and Uma Maheswari. K.., Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)-based nanofibrous scaffolds to support functional esophageal epithelial cells towards engineering the esophagus. J Biomater Sci, Poly, 2014, 25, 574-593.
17. Purushothaman. K, Swaminathan. S and Krishnan UM, Interaction of human smooth muscle cells with nanofibrous scaffolds: Effect of fiber orientation on cell adhesion, proliferation, and functional gene expression. J. Biomed Mater Res, 2015, 103,2236-2250.
18. Purushothaman. K, Kirthanashri. S. V , Dhakshinamoorthy. S , Uma Maheswari .K , and Swaminathan S., Development of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Fibers for Skin Tissue Engineering: Effects of Topography, Mechanical, and Chemical Stimuli, Biomacromol, *2011, 12, 3156–3165*
19. Singh G, Kumari A, Mittal A, Yadav A, and Aggarwal NK., PolyβHydroxybutyrate Production by *Bacillus subtilis* NG220 Using Sugar Industry Waste Water, BioMed Res Int, 2013, doi: 10.1155/2013/952641
20. Urmila , Sridhar.R, and Sehgal. P.K, Biosynthesis and biocompatibility of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) produced by Cupriavidus necator from spent palm oil. Biochem Eng J, 2010, 49 ,13–20.
21. Valappil. S.P, S.K. Misra. S.K, Boccaccini .A.R, Keshavarz.T C. Bucke.C, and Roy.I., Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterized *Bacillus cereus* SPV, J Biotechnol, 2007, 132 , 251–25.
22. Srividya. S. , Polyhydroxybutyrate (PHB) production using agro-industrial residue as substrate by Bacillus thuringiensis IAM 12077 International Journal of ChemTech Research; 2012,1158-11 62.
23. Leda R. Castilho , David A. Mitchell , Denise M.G. Freire., Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation Biores Technol, 2009, 100, 5996–6009.
24. Kulkarni .S.O, Kanekar.P.P , Jog.JP , Sarnaik.S. P, and Nilegaonkar. S.S., Production of copolymer, poly (hydroxybutyrate-co-hydroxyvalerate) by Halomonas campisalis MCM B-1027 using agro-wastes, Inter J Biol Macromole, 2015, 72, 784–789.
25. Artun S, Ipsita R, Tajalli K., Agro-Industrial Waste Materials as Substrates for the Production of Poly(3-Hydroxybutyric Acid) J Biomater Nanobiotechnol, 2014, 5, 229-240

*****