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Study of Bacterial Biopolymer Production by *Bacillus* Species

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A B S T R A C T

Modern world can‘t be imagined without plastics due to their vast applications in contemporary life. All the plastic products which are available in market are made up of Petrochemical based synthetic plastics. Such plastics are much difficult to biodegrade which results in its wise accumulation in environment and affects biogeochemical cycles enormously. However, Bioplastics made up of Polyhydroxybutyrate (PHB) provide a better alternative to life threatening petrochemical plastics. PHB is a natural biopolymer produced by numerous bacteria by using different substrates and easy to extract. Thus on nature’s demand the author was performed an experiment with the aim isolate PHB producing bacteria form natural environment and optimized the PHB producing capability of selected isolates. Among twenty two bacterial isolates, five isolates were showed prominent PHB production at 37°C in presence of glucose and sucrose as a carbon source while peptone a nitrogen source in medium with pH 7.0. The isolates which showed more than 50% PHB production were identified as *Bacillus licheniformis* and *Bacillus cereus*. PHB polymer production by isolate was confirmed by UV spectrophotometer analysis.

Introduction

Plastic materials are now become a most important part of human beings life, unfortunately causing serious environmental problems. Abundant synthetic plastics are accumulating in the environment because of its non-biodegradability. Meanwhile they fragment into smaller microplastics or nanoplastics and enter in food chain which will be life threating to living organisms. This is our responsibility to make sure that we keep plastic within a circular economy but out of the environment.” The plastic accumulation in environment can be reduced by using Biodegradable plastic. It will be possible when researchers and industrialist will focus more on large scale biodegradable Bioplastics production. Varieties of biodegradable thermoplastic polyesters can be used to overcome the problems of plastic

waste accumulation. One of these promising materials is Polyhydroxyalkanoates (PHAs) (Gironi *et al.*, 2011).

In bioplastic production, PHAs has gained major attention due to their structural diversity and close analogy to plastic polymer. (PHAs) are naturally produced during microbial metabolic processes. There are different types of PHAs. PHB is one of the most studied Polyhydroxyalkonates which can be used for bioplastic production. It is linear polyester composed of 3-hydroxybutyrate (3HB) repeating units with the general formula of PHAs. It is the reserved food polymer produced during time of bacterial starvation and provides carbon and energy when external carbon source is exhausted.

PHB production is a promising technology that can change the scenario of plastic waste management. Bacterial PHB is not only easily biodegradable but also biocompatible. It has mechanical properties that are very similar to conventional plastics including polypropylene or polyethylene. Thus it can be used in productions of many domestic and commercial applications such as food packaging, biowaste bags and stationary. Its compatibility with human tissues made it possible to use it in coating of medicine and surgical appliances (Chang *et al.*, 2015).

Both Gram positive and Gram-negative bacteria can produces PHB. Gram negative bacteria belonging to genus *Alcaligenes*, *Ralstonia* and *Pseudomonas*, *Halobacterium*, *Haloarcula* has ability to produced PHA production. While, following are the certain Gram-positive bacterial spp. like *Clostridium*, *Corynebacterium*, *Nocardia*, *Bacillus*, *Rhodococcus*, *Streptomyces* and *Staphylococcus*, showed significant PHB production (Singh *et al.*, 2009). The methods utilized for extraction of Bacterial PHB are also technically and economically feasible. Just there is need of large scale fermenters and extractors for mass production of PHB.

Hameed El-Abd *et al.*, (2017) studied the biodegradable and biocompatible characteristics of PHB produced by *Bacillus thuringienesis* bacterial spp. It showed 4.1 g/L PHB production using 30 g/L Sugar-cane molasses (SCM). The obtained PHB was completely degraded by Natural Soil microorganisms within four weeks.

The *Bacillus* spp. isolated by Getachew *et al.*, (2016) was found to be the best producer of PHB using different types of agricultural waste like sugar cane bagasse, corn cob, teff straw (*Eragrostis tef*) and banana peel as carbon source. The optimum pH, temperature, and incubation period for maximum PHB production by the isolate were 7, 37°C, and 48 h respectively at 150 rpm. The obtained PHB was characterized by FTIR and UV–Vis spectrophotometric analysis. It showed maximum similarity with standard PHB and thus found to be suitable for bioplastic production.

Thus by observing the use of Bioplastics in literature survey, the author decided to work on the isolation of the PHB producing bacteria from environmental samples, produced PHB using suitable media and to determine suitable method for PHB extraction.

Materials and Methods

Sample collection

Soil samples from agricultural field, polluted river and Botanical Nursery located near to Panvel Tahsil, Dist: Raigad (MS) were collected as a source of biopolymers producing bacteria.

A homemade composted soil sample was also used for screening of PHB producing bacteria. Samples were collected by dipping up soil up to 1ft. in clean & dry plastics bags.

Sample Enrichment for PHB producing bacteria

1gm of each soil sample was enriched in 100ml Nutrient broth (NB) Medium. Flasks were incubated at R.T. (28°C ± 2°C) for 48h on shaker at 150 rpm. After incubation, growth was observed in the form of turbidity.

Isolation of PHB producing bacteria

The isolation of PHB producing bacteria was carried on Nutrient agar medium using spread plate method. Enriched samples were first serially diluted up to 10^{-6} dilution.10^{-3}, 10^{-4}, and 10^{-5} dilutions of each sample were used for isolation. Plates of each sample were observed for isolates with different colony characteristics. Isolates were labeled and maintained on Nutrient agar slants at 4°C.

Primary Screening of PHB producing bacteria

Total 21 bacterial isolates were found during isolation. Primary screening was performed to confirm their ability of PHB production. It was carried out by Plate staining method using Sudan black B stain (Shaaban *et al.,* 2012).

The microbial growth on Nutrient agar plates was flooded with Sudan black B stain (0.3 gm Sudan black B dye in 100ml 70% Ethanol) for 30 min. Ethanol used to remove excess stain. The isolates that retained their black color were confirmed as PHB producing isolate.

Screening of PHB production by microscopic staining method

05 isolates showed positive primary screening test on agar plate were subjected to microscopic observation of PHB granules by Burdon's method. Positive Organisms showed in Blue- black color granules with pink cell cytoplasm (Chetia, 2019).

Extraction, confirmation and quantification of PHB produced by different bacterial isolates (Nehra *et al.,* 2015; Swathi *et al.,* 2015; Musa *et al.,* 2016).

PHB extraction was carried out by using solvent extraction method while quantification of extracted PHB was done by using std. curve of Crotonic acid as described by Nehra *et al.,* (2015). 3ml saline suspension of 24h old culture of each isolate with 0.6 O.D. (Optical density) at 600 nm was inoculated in respective 100 ml medium flask. Media was incubated at R.T. (28ºC± 2ºC) on shaker with 150rpm for 48h.The cell growth of each isolate was pelleted at 10,000 rpm at 40°C for 10 min.

The pellet was washed with acetone and ethanol to remove the unwanted materials, resuspended in equal volume of 4 % sodium hypochlorite and incubated at room temperature for 30 min. The mixture was then centrifuged at 10,000 rpm for 10 min. to sediment the lipid granules. The supernatant was discarded, and the cell pellet was washed successively with acetone and ethanol. The pelleted polymer granules were dissolved in hot chloroform and filtered through Whatman No. 1 filter paper (previously treated with hot chloroform). To the filtrate, 10 ml of hot concentrated H_2SO_4 was added, which converts the polymer to crotonic acid. The absorption spectrum of solution was recorded from 200 nm to 500 nm by UV spectrophotometer (Shimadzu UV spectrophotometer: UV-1800). Conc. H_2So4 used as a blank for reading.

The quantity of PHB produced was determined by referring to the standard curve. Standards of Crotonic acid were prepared according to the statement; One gram of PHB is equivalent to 1 gram of crotonic acid (Law *et al.,* 1961).

Identification PHB producing isolates

02 isolates were found to be more efficient in PHB production. Both isolates were identified by their morphological, cultural and biochemical characteristics. The results of all the isolates were compared with Bergeys manual of bacteriology for the identification of each isolate.

Optimization study

Carbon component, Nitrogen component, pH of culture medium and Incubation temperature plays an important role in PHB production. Thus the media was optimized by observing the effect of various nutrient sources on growth of each isolate. The whole experiment was conducted in triplicates and

mean value was taken for analysis. Mineral salt medium (MSM) containing Glucose - 1gm, Peptone -0.25gm, Yeast extract – 0.25gm, NaCl – 0.01gm, KH_2PO_4 – 0.05gm, $MgSO_4$ – 0.02gm, pH – 7, D/W – 100ml was used for optimization studies. The medium was prepared and sterilized at 121°C. A working volume of 100ml MSM medium in 150ml Erlen-meyer flask was employed throughout the study.

Results and Discussion

Isolation of PHB Producing bacteria

Analysis of 04 samples to isolate PHB producing bacteria resulted in isolation of 22 bacterial isolates. From each sample, colonies with different colony characteristics were selected for further experiments. Out of 22, 06Isolates were obtained from Botanical Nursery soil sample, Karajade, 07 isolates were isolated from Agricultural field sample, Kalundre. While 06 isolates were obtained from Polluted soil sample, Gadhi River and 03 isolates were obtained from Composted soil sample. The colony morphology of each isolate from each sample was found to differ in colour, shape, size, margin, surface texture, consistency and elevation. The selected isolates were purified, labelled and stored on Nutrient agar slopes below 15^0C.

Soil and activated sludge serve as a important source of PHB producing bacteria. In present study, the bacteria were isolated form nursery soil, agricultural soil and polluted water samples to determine their ability to produce PHB. Similarly, an attempt was made by Shaaban *et al.,* (2012), to isolate efficient poly-β-hydroxybutyrate (PHB) producing bacteria from different agricultural soil samples in Egypt. They found 10 promising PHB bacterial isolates from 15 soil samples.

Some of the investigators isolated PHB producing bacteria from different low cost carbon sources. Ghanbarnezhada *et al.,* (2013) isolated Biopolymer producing bacteria from some *Brassica* plants such as cabbage, cauliflower and lettuce which were identified as *Pantoea agglomerans*, *Bacillus subtilis*, *Enterobacter* spp. and *Pseudomonas* spp.

In this study, the samples were enriched in sterile nutrient broth medium considering it suitable for growth of all kind of bacteria. Turbidity in the test flasks indicated the growth of soil flora in Nutrient broth while medium control flask didn't showed any bacterial growth. Biradar *et al.,* (2015) also successfully isolated PHB producing bacteria from soil samples like crop, nursery, and fodder fields using sterile Nutrient Broth and Nutrient agar medium.

Primary Screening of PHB producing bacteria by Sudan black B plate staining method and Microscopic method

Sudan black B is a preliminary screening agent for lipophilic compounds like PHB. Direct bacterial colony staining using Sudan black B is a simple method for rapid detection of PHB accumulating bacteria from environment. The colonies unable to incorporate the Sudan Black B appeared white, while lipid producers appeared bluish black (Mascarenhas *et al.,* 2017; Pillai *et al.,* 2017).In current investigation out of 22 isolates, only 05 isolates were found to be PHB producing bacterial species. Isolates with positive agar plate test were again confirmed for PHB production by Microscopic observation using Burdon's staining method. All 05 isolates showed Black colour granules on Pink colour rod shaped bacteria. It indicated that all the isolate has ability to produce PHB.

Extraction of PHB produced by different bacterial isolates

It is generally accepted that microorganisms isolated from a natural environment are poor in nutrient sources. Chandani *et al.,* (2014) stated that some bacteria can accumulate up to 60-80 % of their weight as PHB under nutrient limited conditions. In line with above statement, the selected bacterial isolates in present investigation were also able to

produce PHB in satisfactory quantity using simple Nutrient media containing a single carbon and nitrogen source at pH 7.0.

PHB extraction using solvents like 4% sodium hypochlorite, Acetone and Ethanol was found to be suitable for yielding maximum PHB from cell biomass. PHB is a intracellular product, thus there are basic steps to follow during its extraction which includes pretreatment to disrupt the cell walls, precipitation of excreted PHB from cells and PHB purification. The first step involved cell disruption by sodium hypochlorite (NaClO) to dissolve the cell wall and chlorophyll. Acetone and ethanol were used to precipitate PHB. Finally pure PHB was obtained after hot chloroform treatment of precipitate. This principal of solvent extraction was experimentally explained by Dianursanti *et al.,* (2019) and Requiso *et al.,* (2021).

Many scientist extracted PHB from Bacterial isolates by using above solvent extraction method. Rawate *et al.,* (2012) reported that 5 min. treatment of cell pellet with Sodium hypochlorite gives maximum and accurate accumulation of PHB in production media. The statement was found to be correct during extraction process of current study. The yield of PHB increased when the reaction time of cell lysis step was increased up to 5 min. Aramvash *et al.,* (2018) noted that Hypochlorite extraction method gives higher yield of PHB. It may be due to its more cell permeability and the removal of cell debris and residual lipid from slurry. According these observations it is confirmed that Sodium hypochlorite is one of best cell lysis agent which can be used at large scale extraction of PHB from bacterial isolates.

Qualitative estimation of PHB extracted from selected Bacteria

The residue of extracted PHB from each isolate was confirmed by U.V. spectrophotometer analysis using Crotonic acid as a standard solvent. PHB extracted from selected 05 isolates showed highest wavelength maxima at 235nm which indicates that the extracted residue contains only Polyhydroxybutyrate molecules.

Standard commercial PHB is convert into crotonic acid (brown colour compound) when it reacts with hot Conc. H_2SO_4 (Marjadi *et al.,* 2014). In present study, when extracted residue was treated with hot Conc. H_2SO_4, brown colour compound was observed which showed highest maxima at 235nm similar to std. Crotonic acid. Thus we successfully extracted PHB from selected isolates. Fig.3.1shows the U.V. Spectrum of analysis of extracted PHB from isolates.

Similar results of Successful extraction of bacterial PHB were also reported by many inventers including Marjadi *et al.,* (2014) and Gudmalvar *et al.,* (2014).

Quantitative estimation of PHB produced by different bacterial isolates

The Concentration of PHB present in extracted residue was determined by using std. curve of Crotonic acid (10-150mg/ml). Total production of PHB was obtained within 40 - 130 mg/ml range. Amongst five isolates, two isolates produced more than 100mg/ml PHB, two isolates exhibited up to 90 mg/ml PHB production while one isolate showed only 48 mg/ml PHB production. Total PHB production by each isolate was represented in Table 3.1. Isolate from fertile soil samples showed maximum PHB production as compare to isolates obtained from polluted sample.Fig. 3.2 shows the Std. Curve of Crotonic acid at 235 nm.

Similarly Sharma *et al.,* (2013) observed highest PHB production by bacterial isolate obtained from composting soil samples. Nehra *et al.,* (2015) noted that the isolates obtained from the rhizospheric area of different crops can produced highest amount of PHB. The above observations suggest that the fertile soil flora also has ability to produce PHB.

In present study the isolates were grown in sterile Nutrient broth to quantify the PHB produced by

each isolate. The media was found to be very simple in composition and supported the bacterial isolates to produce maximum amount of PHB. However, Shaaban *et al.,* (2012) quantified PHB production by referring standard curve of Crotonic acid and for quantification they used Luria Bertani broth supplemented with glucose (2%). Similarly, Sujatha *et al.,* (2005) also used LB broth containing glucose 2% as the medium, which favoured PHB accumulation due to higher C: N ratio. It indicates that the bacteria can produce PHB in simple and low cost medium.

Identification of prominent PHB producing isolates

The isolates showed maximum PHB production were identified as species of *Bacillus* genus on the basis of Bergey's Manual of Systematic Bacteriology and Bergey's Manual of Determinative Bacteriology6A and 2D isolates showed large creamy colonies on nutrient agar. Both isolates were found to be Gram Positive rod shaped spore forming bacteria.

Both could hydrolyse Starch, produce Catalase enzyme, ferments glucose and sucrose sugars. Both showed positive results for Motility test. Isolate 6A showed Positive results for Methyl red and negative results for Indole, Vogus Proskaur and Citrate utilization test. However, isolate 2D showed Positive results for VP and Citrate and Negative results for Indole and Methyl red test. Isolate 6A showed growth at 50°C while 2D could not. These Biochemical results confirm that the isolate 6A is *Bacillus licheniformis* and 2D is *Bacillus cereus.*

The results of biochemical characteristics of both isolates were compared with results obtained by Oyeleke *et al.,* (2011) and Umayaparvathi *et al.,* (2013) through which the author confirmed the bacterial identity as a *Bacillus lichniformis* and *Bacillus cereus*. Similar to our results, Narayanan *et al.,* (2020) also identified *B. cereus* NDRMN001 from polluted lake soil samples which has the potential to yield 33.19 gm/ L of PHB with fine quality and quantity in a short duration by utilizing cheap nutritional factors.

Optimization of PHB production process of prominent isolate

All the parameters (pH, temperature, carbon sources and nitrogen sources) showed significant effect on growth of 6A and 2D isolates. The cell biomass and PHB yield of both isolates at each optimized factor was measured and represented graphically.

There are many reports which are suggests that bacteria starts PHB synthesis in nutrient limiting conditions as compared to its favourable growth conditions. Similarly in present study, both the isolates did not show PHB granules production after 24h of incubation. The cell biomass obtained after 48h and 72h of growth showed the presence of PHB granules. It could be because of nutrient exhaustion after 24h. Thus during optimization study, the PHB yield at each parameter was measured after 72h of incubation time.

Both isolates showed maximum PHB Yield in presence of glucose as carbon source. Peptone and Ammonium sulphate was found to be more suitable nitrogen source for higher PHB yield while there was slight reduction in PHB yield in presence of Ammonium chloride. The most suitable temperature for maximum PHB yield was R.T. (28°C±2) and pH was 7.0.

Both the *Bacillus* spp. showed maximum PHB in presence of Glucose as carbon source. Similar observations were reported by Alshehrei, (2019). They found that the Glucose is the best carbon source while NH_4SO_4 is the best nitrogen source for PHB production by *Bacillus* spp. Lee *et al.,* (1999) and Shah, (2012) observed maximum PHB yield by *Bacillus* spp in presence of yeast extract and peptone as a carbon source.

Table.1 Total PHB production by each isolate

Isolate	O.D. at 235 nm	PHB concentration (mg/ml)
1A	0.43	48
6A	0.82	120
2B	0.68	89
3C,	0.67	85
2D	0.87	127

Fig.1 U. V. spectrum of PHB extracted from *Bacillus licheniformis* and Std. crotonic acid

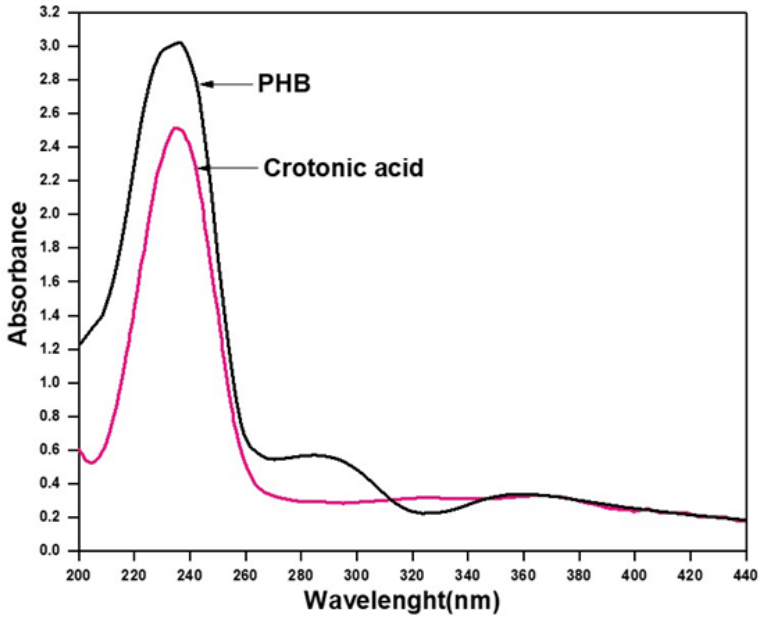
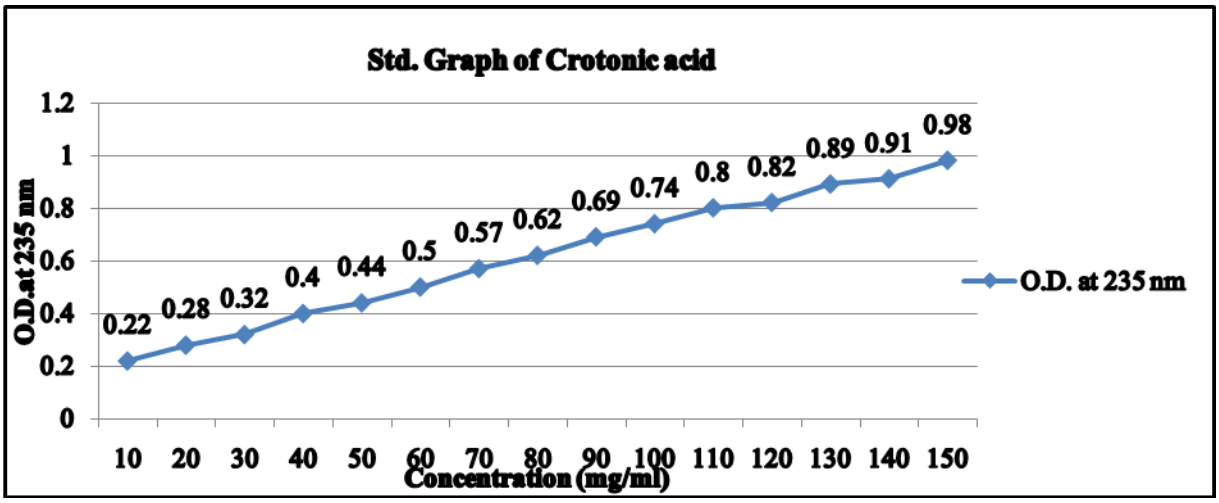


Fig.2 Std. Graph of Crotonic acid (10-150mg/ml)



The present study concluded that the isolated bacteria from different soil samples have capacity to produce maximum Polyhydroxybutyrate granules. Bacillus cereus and *Bacillus licheniformis* will offer considerable advantages in the large scale production of Polyhydroxybutyrate polymer at industrial level. The solvent system used for extraction of PHB was easier to handle and economically viable to apply at large scale production of PHB. The experimental work can be explore for production of Polyhydroxybutyrate using appropriate bioreactor and designed media fulfilling optimum parameters obtained during study.

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