Large Scale Production of PHB And Comparative Analysis of Production from Halophilic and Non Halophilic Strains

Sonali Saini[1]*Pallavi Koundal[1]
[1]Assistant Professor, Department of Biotechnology, Modern Group of Colleges, Mukerian, Punjab, India.

Abstract - Polyhydroxybutyrate (PHB) produced by bacterial species (Halomonas meridiana). Polyhydroxyalkanoates represent a potent replacement to synthetic plastics because of their biodegradable nature. This study aimed to screen bacteria isolated from agriculture and vegetable soil sample. Among them, two strains halophilic and non halophilic strains showed positive results with phenotypic and genotypic methods. Phylogenetic analysis, based on the 16S rRNA gene, indicated that polyhydroxyalkanoate (PHA)-producing bacterial isolates IDSEQ01 were related to *Halomonas meridian*.Under nutritionally optimal cultivation conditions, polymers were extracted from these strains and were determined by gravimetric analysis yielding PHA production of 30% and 20% of cell dry weight. In conclusion, optimization of PHB production from inexpensive industrial wastes and carbon sources has considerable interest for reducing costs and obtaining high yield.

Index Terms - Biosynthesis, PHB, Bacterial fermentation, Biosynthetic polymers, Polyhydroxyalkanotes

I. INTRODUCTION

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate(PHA), is a polymerbelonging to the polyesters class that are of interest as bio-derived and biodegradable plastics. PHB is produced by microorganisms such as *Ralstonia eutrophus or Bacillus megaterium* apparently in response to conditions of physiological stress. Plastics being xenobiotic are recalcitrant to microbial degradation [1]. Plastics have become an important part of modern life and are used in different parts of operations like packaging, building materials, consumer products and many more. Presently plastic and synthetic polymers are mainly produced by using the petrochemical material that cannot be decomposed easily [2]

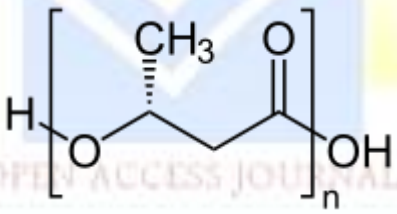

Fig1. Structure of poly-(R)-3-hydroxybutyrate (P-3-HB), a polyhydroxyalkanoate.

The use of non biodegradable plastics can act as control on bringing down the level of environment pollution; hence biodegradable plastics have emerged as a useful alternative to overcome the environmental pollution. PHB is a carbon and energy source for some bacteria under stress conditions. PHB is found to accumulate in large number of microorganisms as reserve food material e.g. *Ralstonia eutrophes, Azotobacter beijerinckia, Bacillus megaterium, Pseudomonasoleovorans*and various nitrogen fixing microorganisms. Due to similarity in physical property with synthetic polymer, it is possible to use PHB in high scale. But the high production cost of PHB, restrict its wider application .Therefore alternative strategies such as optimization of the temperature, time, pH, carbon, nitrogen sources and other growth conditions and easier downstream processing methods are

required for reducing the cost. The first example of PHAs to be discovered was polyhydroxybutyrate (PHB). Isolated and characterized PHB from *Bacillus megaterium*.[3].

Polyhydroxyalkanoates (PHAs) are polyesters synthesized by various microorganisms, such as *Ralstonia eutropha, Alcaligenes latus, Aeromonas hydrophila*, *Pseudomonas putida*and *Bacillus sp.* The PHA types, such as polyhydroxybutyrate (PHB), poly(hydroxybutyrate-co-hydroxyvalerate(PHBV),poly(hydroxybutyrate-co hydroxyhexanoate) (PHBHHx) and polyhydroxyoctanoate (PHO) are frequently studied for biomedical applications including tissue regeneration devices, repair devices, repair patches and sutures [4].

PHB is accumulated inside a variety of microorganisms under appropriate conditions such as limitation of nitrogen, calcium, magnesium, iron or essential vitamins. PHB has also been found in numerous heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria, actinomycetes , cyanobacteria and many other prokaryotes.

The physical and mechanical properties of these copolymer such as stiffness, brittleness, melting point, glass transition temperature, and resistance to organic solvent can change considerably as a function of the monomer composition (5].

Poly (3-hydroxyalkanoates) are classified by the length of their monomeric units:

(1) Short-chain-length

(2) Medium-chain-length

(3) Long-chain-length PHAs may contain any combination of the monomeric unit

PHA-producing genera and species, *Escherichia coli* (Lee et al., 2005) and *Cupriavidus necator* (formerly *Wautersia eutropha, Ralstonia eutropha,* and *Alcaligenes eutrophus* (Vandamme, 2004).

Various types of natural sources and synthetics lab medium are used as substrates for PHAs production such as palm oil, glycerol, wheat and malt [6]. MS medium, M9 medium and DSC-97 medium . Poly-hydroxybutyrate is the most common biodegradable polymer and a promising alternative to synthetic non-degradable plastics. The literature studied for the research is as under:

1 Biosynthesis of PHB

PHB biosynthesis is the product of microbial metabolism and in most bacteria, such as *Alcaligenes eutrophus*, PHB is synthesized in a three-step reaction starting with acetyl-

CoA when cultivated on carbohydrates, pyruvate, or acetate. Two acetyl-CoA molecules are coupled to form acetoacetyl-CoA in a condensation reaction catalyzed by 3-ketothiolase. The product is subsequently selectively reduced to (R)-3-hydroxybutyryl-CoA reaction catalyzed by NADPH-dependent acetoacetyl-CoA reductase. Finally, PHB is synthesized by polymerizationof (R)-3-hydroxybutyryl-CoA molecules by the PHB synthase.

Poly-β-hydroxybutyrate (PHB) is a natural polymer which is produce intracellularly by various microorganisms [7]. PHB exists in the cytoplasmic fluid in the form of crystalline granules about 0.5 μm in diameter and can be isolated as native granule. The lipid inclusion is accumulated by many bacteria when they enter the stationary phase of growth to be used later as internal reserves of carbon and energy.

The main advantage of PHB is that, due to their biological origin, they are degraded naturally and completely to carbon dioxide and water under natural environment by the enzymatic activities of microbes. It was observed that a marine isolate was able to produce nearly 40% of PHB granules as nutrient resource[8]

2 PHAs Biosynthesis in Recombinant E.coli

Polyhydroxyalkanoates (PHAs) are microbial polyesters that can be used as completely biodegradable polymers, but the high production cost prevents their use in a wide range of applications. Recombinant *Escherichia coli*strains harboring the *Ralstonia eutropha*PHA biosynthesis genes have been reported to have several advantages as PHA producers compared with wild-type PHA-producing bacteria. However, the PHA productivity obtained with these recombinant *E. coli*strains has been lower than that obtained with the wild-type bacterium *Alcaligenes latus*. The three PHA biosynthesis genes form an operon with the order PHA synthase, b-ketothiolase, and reductase genes and were constitutively expressed from the natural promoter in E. coli. Recombinant *E. coli* strains harboring the *A. latus*PHA biosynthesis genes accumulated poly(3-hydroxybutyrate) (PHB), a modelPHA product, more efficiently than those harboring the *R. eutropha*genes. With a pH-stat fed-batch culture of recombinant E. coli harboring a stable plasmid containing the *A. latus*PHA biosynthesis genes, final cell and PHB concentrations is high. This improvement should allow recombinant E. coli to be used for the production of PHB with a high level of economic competitiveness.

3) Poly-β-Hydroxy Butyric Acid Production, Degradation and its Optimization using Alcaligenes Sp. and Pseudomonas Sp.

Poly-β-Hydroxy butyric acid (PHB), was produced by fermentation technology of two-stage cultivation process. The organism used is *Alcaligenes sp*. and *Pseudomonas sp.* At first, the various isolates were cultured in the nutrient broth medium at 30°C and 200 rpm for 24 hr. After incubation, the 2 ml of culture was taken to inoculate the flask containing 200 ml of sterile production medium, which is limited of nitrogen source. The produced PHB was extracted using sodium hypochlorite digestion process followed by solvent extraction technique. PHB was identified using Thin Layer Chromatography and was quantified spectrophotometrically at 235 nm. The production of PHB by *Alcaligenes sp.*is found to be high as compared with *Pseudomonas sp.* The produced PHB was characterized using IR spectroscopy. The extracted PHB was tested for its degradation by overlay method. The PHB was finally subjected to film preparation using Poly ethylene glycol (PEG).

4) Screening and Evaluation of Polyhydroxybutyrate-Producing Strains from Indigenous Isolate Cupriavidus taiwanensisStrains

This study developed a system for screening strains in order to optimize PHA production in *Cupriavidus taiwanensis* and *Pseudomonas oleovorans*. Sudan black B staining, Infrared (IR) and Gas Chromatography (GC) analysis indicated that the best strain for PHA synthesis is *C. taiwanensis*. Cultivation of *C. taiwanensis* under pH of 7 at 30 °C, and at an agitation rate of 200 rpm, obtained a PHB content of 10% and PHB production of 0.14 g/L. Optimal carbon/nitrogen ratio for PHB production was also determined. PHB content of

58.81% and a PHB production of 2.44 g/L when the carbon/nitrogen ratio of 8/1 is select for *C. Taiwanensis*. A two-stage fermentation strategy significantly enhanced PHB content and PHB production.

II. MATERIALS AND METHODS

1) Collection of Samples

Soil sample were collected from different sites of agriculture area situated at Jalandhar, Gurdaspur and Himachal Pardesh, district Bilaspur as shown in Figure 1 and figure 2. The samples were stored in sterile plastic bags at 4 ^{o}C and were transferred to laboratory.

Fig. 2 Soil sample were collected from vegetable root soil.

Fig. 3 Soil sample were collected from Agriculture area.

2) Isolation of producing Bacteria

Soil samples after serial dilution spread on the nutrient agar medium and appearance, unknown colonies observed.

Agriculture soil samples after serial dilution spread on the nutrient agar medium and kept for one to two weeks incubation. Growth of pure colonies after streaking. Appearance of yellow and orange colour colonies of unknown bacterial isolates. The best growth of pure isolate colonies on 1M and 2M 3M Nacl concentration of the nutrient Broth.

3) Isolation of PHB Producing Bacteria

Six sterile test tubes were taken and serial dilution was done. Spreading of samples at dilution $10^{-3,}$ 10^{-4} and 10^{-5} were done on Nutrient agar medium using L- shaped spreader All the petriplates were incubated at 37 °C for 24-48 h. Pure colonies were screened out by streaking them on Nutrient Agar medium at different molar Nacl concentration i.e. 1M, 2M and 3M.

Optimization of Culture Conditions for maximum PHB production is as under:

1) Media optimization for PHB production

The four mediums i.e., nutrient broth (NB), DSC-97medium, M9 medium and YEM medium are used for the production of PHB and growth of isolate. After 48 hrs of incubation at 37^{o}C, the bacterial growth and PHB production are tested.

2) Media composition

a) Composition of the nutrient broth is shown in Table 1

Table 1 Composition of the nutrient broth

Constituents	Quantity in g/L
yeast extract	1.5
beef extract	1.5
Peptone	6.0
Nacl	as per requirement
Distilled water	1.0 L
pH	7
$FeSO_6.7H_2O$	6.99g/50ml

3)PHB Production

3.1) Bioreactor Experiments

The fermentation carried out aerobically on a bench scale fermenter manufactured by BioGen. Which consists of different medium solution was sterilized in the autoclave at 15^{o}C with automatic pH 2 controller, automatic dissolved O_2 controller, $2CO_2$ controller automatic temperature controller, foam controller and multi-channel peristlaic pump. The PHAs producing bacteria were grown in the bioreactor as batch, two-stage batch and fed-batch cultivation.

3.2) Fermentation Condition for Optimized Media

The optimized media is supplemented with all the other optimized parameters such as pH, temperature, molar salt concentration, incubation time, carbon source and nitrogen source for the large scale fermentation. The objective of doing fermentation on optimized parameters is to maximize the bacterial growth for large scale of production of PHB.

3.3) Characterization of PHB

1) IR analysis of PHB

One mg of sample is ground well with 10 mg of spectral pure anhydrous potassium bromide crystals. The powder is make into pellet for IR analysis. The relative intensity of transmitted light energy is measure against the wavelength of absorption on the region using IR Spectrum (Prabakaran,2006).

2) GLC Analysis of Extract PHB

Sample (200mL) were analysed for cdw and PHA production Aliquots (22mL) were centrifuged at 4000 rpm for 30min. The pellet was washed with 10-15 mL saline (0.9% Nacl) and recentrifuged. The pellet was dried at 85^{o}C for 36h and weighed to estimate cdw. After the propanolysis of bacterial dry cell extract, the heavier phase containing chloroform solution with the esters of propanol and beta hydroxyacids from PHA hydrolysis were analysed by gas chromatography using 10% Reoplex 400, dimethylpolysiloxane capillary column DB-1 and flame ionization detector (FID). Poly -3-hydroxybutyrate (Fluka Chemika, USA) and Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (Sigma- Aldrich) are used as standard.

III. RESULTS AND DISCUSSION

Halomonas meridiana species is isolated from soil sample**.** The culture conditions of isolated strains were further optimized for the production of PHB. It was observed that at 150 rpm, pH8 and 3M in nutrient broth media outcomes were maximum. Black color spots showed the presence of PHB granules.

IV. CONCLUSION

1 Isolated strain was rod shape i.e gram positive, and was determined by simple staining and Gram staining respectively.

2 Screening of PHB production by the Sudan Black B staining was performed. The granules were inside the bacterial cytoplasm and were stained in black colour by the Sudan Black B dye.

3 The best growth of isolated strain and *Cuprividus nector* MTCC 1472 was on the nutrient broth, glucose 2%, incubation time 72 hours, temperature 37^{0}C , shaking speed 150 rpm and pH 8.

4 The black spots were shown by TLC (thin layer chromatography). The R_f value was observed. The travelled distance between solvent from bottom to top was 10 cm and travelled distance between PHB sample was 7.1 cm from bottom to top on TLC plate.

5 Characterization of PHB by FTIR technique was done. Infrared (IR) spectra of pure PHB shows mainly two intense absorption bands at 1643.41 and 1564.32 cm^{-1}, corresponding to C=O and C-O respectively. IR was carried out for the PHB produced from nutrient broth, the peak value was observed for the isolate strain were 1568.18cm^{-1} and 1417.73cm^{-1}.

6 Genomic DNA of isolated strain was performed successfully and band of DNA was shows on UV illuminator.

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