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Polyhydroxybutyrate (PHB) Production and Optimization using *Staphylococcus Gallinarum* and One More Soil Isolate

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Abstract
In this present study PHB producing strains were isolated from different sites i.e. agriculture soil, oil contaminated soil, beach soil etc. Microbes were screened for PHB production by Sudan black staining and five isolates were found to produce good amount of PHB. All five strains were morphologically and biochemically characterized. Isolates were inoculated in fermentation medium for 48 hours at 37°C and pH 7. The PHB produced was extracted and quantified. Further study involved two strains S7 and S12 based on their good PHB production capacity. They were further optimized for different media parameter such as pH, carbon and nitrogen sources. For bacterial isolate S7 fructose, yeast extract and pH 7 were found optimum carbon source, nitrogen source and pH respectively whereas for bacterial isolate S12 sucrose, NH_4Cl and pH 7 were found optimum carbon source, nitrogen source and pH respectively. With physical mutagenesis experiment isolate S7 gave increase in PHB production. Overall S12 was found very good PHB producer and identified as *Staphylococcus gallinarum* strain S533_4 based on 16SrDNA sequencing and BLAST and phylogenetic analysis.

Keywords
PHB, Bioplastics, Minimal Davis medium, PHB production

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INTRODUCTION:
Plastic materials have become an integral part in our life. Plastics are inexpensive, strong, durable, corrosion resistant materials, with high thermal and electrical insulation properties [1]. They are widely applicable in packaging films, wrapping materials, shopping and garbage bags, clothing, fluid containers, toys household and industrial products and building materials. Improper disposal of plastic materials is a significant source of environmental pollution, potentially harming life. The plastic sheets or bags do not allow water and air to go into earth which causes infertility of soil, preventing degradation of other normal substances, depletion of underground water source and danger to animal life. In seas also plastic rubbish from ropes and nets to the plastic bands from beer packs chokes and entangles marine mammals [2].
Bioplastics are bio-based, biodegradable plastics with almost similar properties to synthetic plastic. Bioplastics are the polymers that are present in or created by living organisms. The term bio based means the material is partly derive from biomass (plants), and also from variety of sources like

polysaccharides, lipids and also proteins. These can be easily degradable in to safe natural compounds within a short period of time [3]. Microorganisms provide a source of bio plastics and biopolymer (polysaccharides) from renewable sources. Although currently considerably more expensive than plastic derived from petrochemicals, bacteria have proved capable of yielding bio plastics with comparable properties. They have the additional advantages of being biodegradable. Considerable effort is also being applied to the discovery of new bacterial sources of polymers with different properties.

Some of Bioplastic materials under development include polyhydroxyalkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides and copolymer and blends of starch. Among this various biopolymer, polyhydroxyalkanoates (PHA) provide good and fully degradable alternative to synthetic plastic. PHA properties are similar to that synthetic plastic. It is synthesized by bacteria under unbalanced growth conditions and accumulate intracellular as carbon and energy source. PHA can be easily degraded aerobically by microorganisms in to CO2 and H2O [4, 5]. PHB is the best known polyhydroxyalkanoates and alternative source of plastics. PHB existing in the cytoplasmic fluid in the form of crystalline granule about in 0.5 diameter, hydroxybutyrate is connected by ester linkage and form PHB. This can be extracted from the cells as native granule or by solvent extraction [6]. Many bacteria including those in the soil are capable of PHB production and breakdown. A series of enzymes, synthesizes or depolymerizes are implied in biosynthesis and biodegradation of PHB and other PHA [7].

PHB is produced intracellularly by various organisms such as *Bacillus megaterium, Palstonia eutrophus, Cupriavidus necter, Rhizobium spp., Azotobacter spp., Pseudomonas spp.,* etc. under physiological stress conditions. These bacteria can accumulate up to 60-80% of their weight as PHB under limiting nitrogen substrate and in the presence of an abundant source of carbon [8].

MATERIALS AND METHODS:

1. Sample collection and isolation:

Soil sample was collected from different sites like Agricultural site soil, oil contaminated site soil, laboratory site soil, River and beach site soil etc. Around 1.0 g of sample was serially diluted in sterile distilled water and plated onto nutrient agar plates. Plates were incubated at 30°C for 24 hours. Colony characteristics were selected, and Gram staining was performed, various colonies of different morphologies were individually picked and were screened for PHB production.

2. Screening of PHB:

Bacterial isolates were cultured for 2-3 days at 30°C in Minimal Davis broth medium (K_2HPO_4-7g, KH_2PO_4-2g, Sodium citrate-0.5g, $MgSO_4$-0.1g, Dextrose-1g, $(NH4)_2SO_4$-1g was added in 1L of water and pH was adjusted to 7.0±0.2) plates and to screen PHB producing bacterial isolates Sudan black B staining was performed [8]. All the chemicals were of LR grade and purchased from Qualigens Fine Chemicals Pvt. Ltd.

3. Quantification of PHB production and selection of isolates:

All the Sudan Black B positives isolates were inoculated in Minimal Davis Medium (MDM) broth and incubated for 48 hours at 30°C and subjected to quantification of PHB production as per the method of Jhon and Ralph [9]. 50ml fermented MDM broth of each isolate was centrifuged at 10,000 rpm for 10 min and the pellet was washed with equal volume of acetone and ethanol to remove unwanted materials. The pellet was resuspended into 10ml of 4% of sodium hypochlorite and incubated at room temperature for 30 min. The whole mixture was again centrifuged, and the supernatant was discarded. The cell pellet containing PHB was again washed with equal volume of acetone and ethanol. Finally, the pellet containing the polymer granules were dissolved in hot chloroform.

4. Assay for selected bacterial isolates:

Chloroform containing PHB was treated with 5 ml concentrated sulphuric acid and boiled at 100°C for 10 min. Addition of sulfuric acid converts the pellet in to crotonic acid which is brown in color. The optical density of sample was read at 235 nm using Systronics double beam UV-Vis spectrophotometer. Standard was performed using crotonic acid (100µg/ml) given by Hiremath et al [10]. and used to estimate concentration of PHB. The concentration of crotonic acid obtained from the extracted broth corresponds to the concentration of PHB.

5.Optimization of Cultural Parameters for maximum PHB production:

Different factors affecting PHB production were studied for selected bacterial isolates. Effect of different carbon sources like fructose, sucrose, and maltose (1.0 g/l), nitrogen sources like ammonium chloride, urea and yeast extract (1.0 g/l) and pH 6, 7 and 8 were studied on two isolates using MDM broth and 48-hour incubation at 30°C. PHB production assays were performed in triplicate every time.

6.Effect of UV on PHB production:

Both isolates were grown in 10ml of nutrient broth. 5ml of culture was centrifuge at 15000 rpm for 10 minutes. Supernatant was discarded and pellet was washed with normal saline and again centrifuged. Pellet was taken out in sterile petriplate and exposed to UV rays for 30 seconds time and inoculated in to 100ml MDM broth. PHB produced was quantified.

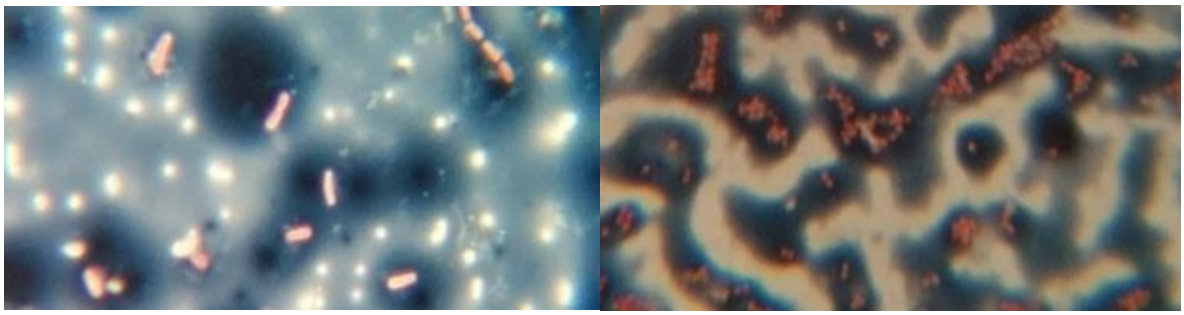
7.Identification based on 16SrDNA sequencing

One selected isolate was sent to saffron Life science for sequencing of 16srDNA sequence. The sequence obtained was further identified on the basis of BLASTn - the tool of NCBI (National Centre for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov/). Maximum target sequence value was set at 10 and rest all parameters were kept default. All 10 sequences were retrieved from the database of NCBI. These sequences were aligned by Clustal Omega – the tool of EMBL (European molecular Biology Laboratory) to get multiple sequence alignment and Phylogenetic tree (https://www.ebi.ac.uk/Tools/msa/clustalo/).

RESULTS AND DISCUSSION:

1.Isolation of bacteria and screening of PHB producer isolates:

From different soil samples about 15 bacteria were isolated. Among 15 isolates, 5 isolates (1 Gram negative rod, 3 Gram positive rods and 1 Gram positive cocci) showed positive Sudan Black B staining and it was confirmed that these isolates were able to store lipid as granules. These 5 isolates were S1, S3, S7, S8 and S12. Granules were stained bluish-black, whereas the bacterial cytoplasm was stained pink in color (Fig. 1).



Isolate S7 **Isolate S12**

Fig. 1 Microscopic appearance of isolates with Sudan Black staining

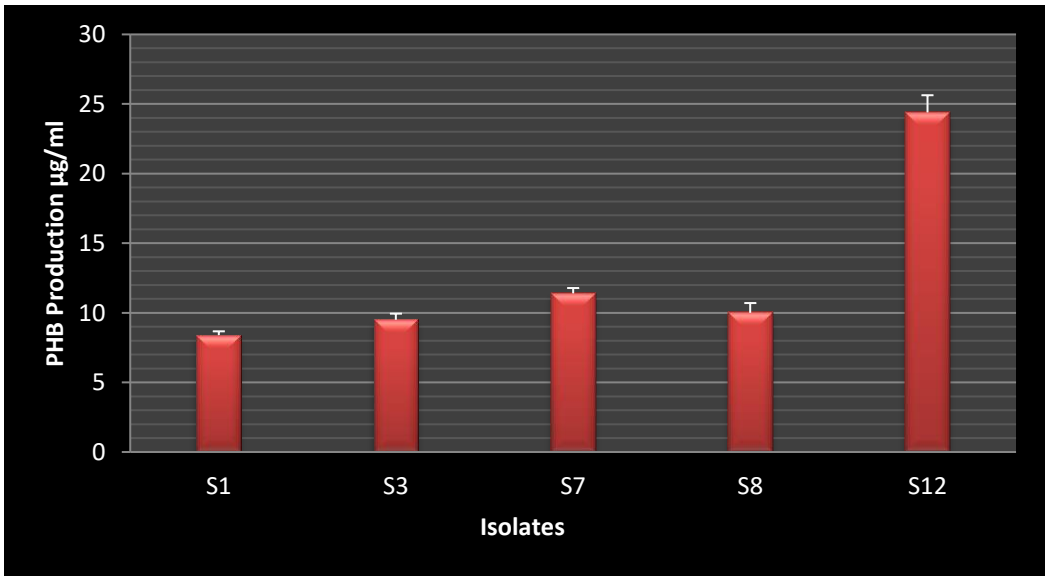


Fig. 2: PHB production given by selected isolates

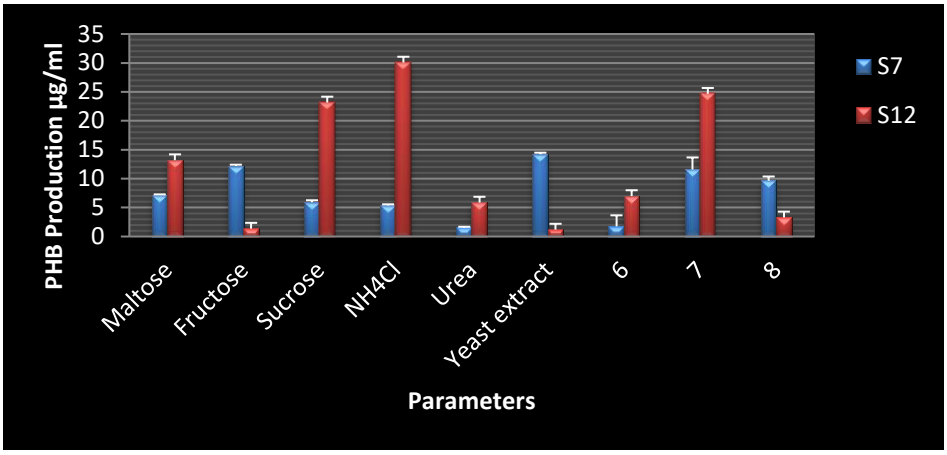


Fig. 3: Results of effect of various C, N Sources and pH on PHB production

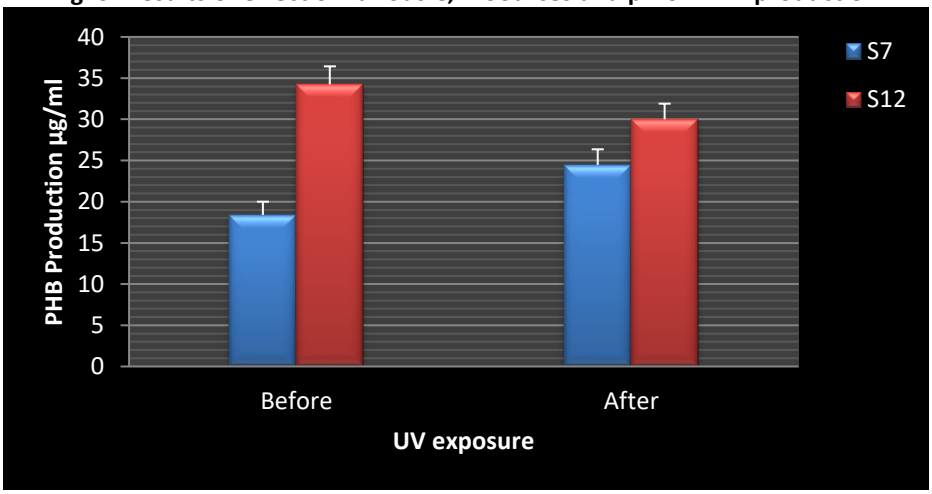


Fig. 4: Effect of UV induced mutation on PHB production

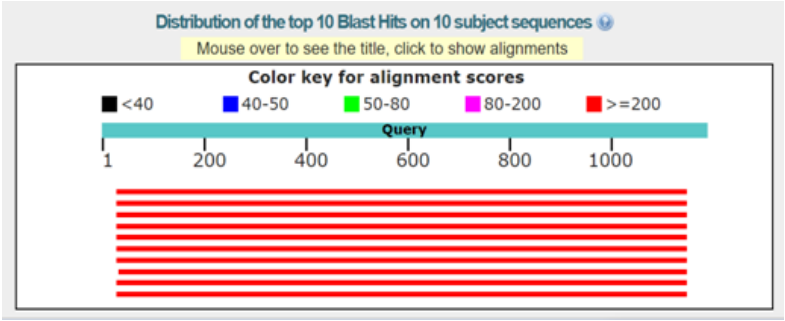


Fig. 5: BLAST analysis of 16srDNA sequence of an isolate S12

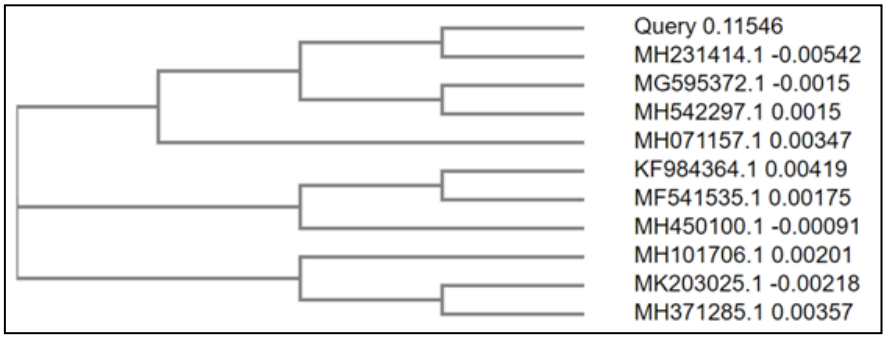


Fig 6: Results of Phylogenetic analysis of query sequence

2. Results of quantification of PHB production and selection of isolates:

From the quantification analysis the bacterial isolate S7 and S12 give maximum concentration of PHB.11.4 and 24.4µg/ml respectively. They were selected for further studies (Fig. 2).

3.Results of optimization of cultural parameters for maximum PHB production:

Carbon sources are important as they serve three different functions within the organisms: biomass synthesis, cell maintenance, and PHA polymerization. Isolate S7 and S12 were optimized for different carbon and nitrogen sources that give maximum production of PHB. In one study researchers found that among the carbon sources tested with the strains BBKGBS6 and BBKGBS27 glucose was found to be the preferred substrate for polymer accumulation. In this study S7 and S12 gave maximum concentration of PHB using Fructose and sucrose as a carbon source respectively [11]. Moreover, nitrogen is an important requirement for nucleotide and amino acid synthesis. S7 and S12 gave maximum concentration of PHB using Yeast extract and NH_4Cl respectively. S7 and S12 were subjected to grow in medium containing pH 6, pH 7 and pH 8 and optimum production of PHB was given by both S7 and S12 at pH 7 (Fig 3). In 2013, Panigrahi and Badveli also reported pH 7 as optimum for PHB production [7].

4. Result of effect of UV light induced mutation:

Mutagenesis is a reliable and cost-effective method to increase PHB yield. Various mutagens like Ultraviolet rays (UV), Acridine Orange are used to mutate some bacterial strains for the production of PHB [12, 13]. Since this method is cost effective it can lead to the production of PHB at lower cost and increase in production levels. Various microbial strains when mutated by these mutagens can lead to the enhanced production of PHB leading to an overall decrease in cost [14]. S7 and S12 were subjected to UV radiation for 30 seconds to induce mutation so as to increase PHB production. S7 showed increase in PHB production after UV exposure from average 18.23µg/ml to 24.3µg/ml, whereas S12 showed decrease in PHB production from 34.43µg/ml to 29.53µg/ml. In one study conducted regarding effect of mutagenesis on PHB production, UV mutagenesis showed almost 2-fold increase in PHB yield in case of *Bacillus flexus,* whereas chemical mutagenesis with acridine orange did not explain any significant enhancement in PHB yield [15].

5.Result of identification of an organism based on 16srDNA sequence and BLAST:

From the above result of 16srDNA sequence the isolate S12 was identified as *Staphylococcus gallinarum* with 95% similarity. Based on the multiple sequence alignment and phylogenetic tree the organism was found closely related to the *Staphylococcus gallinarum* strain S533_4, with accession number MH231414.1.

CONCLUSION:

PHB is a highly strategic material due to its various properties, degradation values, biological origin and biocompatibility. All over the world researchers are continuously striving to increase its production from the existing bacterial strains as well from the new strains [15]. The current study revealed the presence of many PHB producers in different type of soil, which can be used for production of bioplastics in both laboratories as well as at industrial scale. When the PHB production capability was compared S7 and S12 were found to have great PHB production potential. Isolates were tested for optimum carbon & nitrogen sources and pH. Mutagenesis did not show any significant change in our study, but it was only performed after giving 30 second UV radiation. Efforts for the mutagenesis and increase in PHB production must be made during further study. Isolate S12 with best PHB production potential was identified as *Staphylococcus gallinarum* strain S533_4. Hence, the continuous search from the various environmental conditions may provide some more suitable isolates and their genetic modification, can lead to efficient PHB production for commercial use.

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