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# Study of Polyhydroxybutyrate producing *Bacillus*sp. isolated from Soil

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## Abstract

*Poly-ß-hydroxybutyrate (PHB), one of the polyhydroxyalkanoates is the most popularly used bioplastic. These, being biodegradable and biocompatible polymers, are accumulated as energy reserve granules by many microbia intracellularly under carbon rich and nutrient starving conditions. PHB has proved itself as a promising alternative to non degradable plastics. PHB as bioplastics serves advantage of biological origin and can be completely degraded by variety of microorganisms. In the present study, different PHB producing microorganisms were isolated from soil using E2 medium and its rapid screening for PHB production was performed by Sudan Black B dye plate assay. The PHB accumulators were then subjected to spectrophotometric quantitation method and the highest accumulator was identified and characterized. Isolate KOL IV showed maximum accumulation of PHB and was optimized for its ability to accumulate maximum amount of PHB. It was identified as one of the Bacillus species and could finally accumulate 48% of PHB against dry cell mass after optimization.*

**Keywords:** Polyhydroxybutyrate, bioplastics, optimization, biopolymer, biodegradable, biocompatible.

## Introduction

It's been a problem with the concern to degradation and management of plastics worldwide. The non-degradable synthetic plasticshave proved itself havoc for the environment[1]. Plastics are the xenobiotic compounds and are recalcitrant to microbial degradation[2]. This has directed the scientists towards the alternative plastic sources that are environmentally friendly, biologically degradable under appropriate conditions, and can be obtained from cheap sources such as waste products, microorganisms[3]. Bioplastics, alternative to the synthetic plastics, are the polymers produced by many microorganisms and are in turn are biodegradable[4]. Polyhydroxyalkanoates (PHA) are the polyesters that are being synthesized by certain gram positive as well as gram negative microorganisms accrued intracellularly as energy storage particles and can account for around 90% of dry cell mass in nutrient scarce conditions[5]. Polyhydroxybutyrate (PHB) is the best categorized PHAs[1]. PHB has its application in different directions like, packaging material, agricultural and construction materials, sanitary goods, automotive interior materials, electrical devices, bottles and containers, etc.[6]. It can be moulded and spun into fibers, can be polymerized into plastics[7]. It has even shown its steps in the medical applications, pharmaceutical and chemical industries[8]. PHB was first reported to be produced in *Bacillus megaterium*[9]. Many other such organisms were spotted and isolated with their property of producing PHB as reserve food material, *Bacillus cereus, Alcaligeneseutrophus, Azotobacterbeijierinckia, Pseudomonas oleovorans,* etc[10], *Rhizobium* and *Bradyrhizobium* species[11]. The quantitation of the PHB being produced in the organisms can be done by various analytical ways. Use of GC for quantitation was explained by Comeau et al.[2]. FTIR, DSC, NMR was adopted as a quantitation method by Raveendran et al.[13]. The organisms can be optimized for various physiological and nutritional requirements for the maximum production of PHB by the organisms[14]. This PHB can be extracted from the organisms by solvent extraction methods[15]. The molecular advancement has led to the cloning of the gene synthesizing PHB into the recombinant *E.coli*[16,17], but still the scope continues in the search of a better organism, an economical strain.

## Material and Methods

**Isolation of microorganisms from soil:** Organisms were isolated from soil samples of different regions of Madhya Pradesh on E 2 agar medium and were purified[2,7,18].

**Screening for the production of PHB:** Organisms were screened by plate assay method with Sudan Black B dye (0.02% solution in ethanol)[18,19].

**Quantitation of the PHB production by the positive isolates:** For the quantification of the PHB produced by the organisms, the PHB was first extracted from the cells and then quantified by spectrometry. A method described by Jhon and Ralph[12,20].

The activated culture was centrifuged and the pellet suspended in 4% sodium hypochlorite solution. Next centrifugation leads to treatment with chloroform and sulfuric acid and finally the spectrophotometric analysis at 235 nm.

**Characterization of the highest producer:** The best producer was subjected for gram's staining leading to the different biochemical tests for their characterization viz. Sugar

fermentation, $H_2S$ production, starch and lipid hydrolysis, gelatin liquefaction, acid production, catalase test, etc[21].

**Optimization:** Factors affecting PHB production were optimized for the selected organisms. After incubation, quantification was carried by the method described by John and Ralph (1961). Effect of different carbon sources, different nitrogen sources, different pH and temperature and incubation time and the different C:N ratio were studied[22-25].

**Effect of carbon sources on PHB production:** The isolates were activated in 100 ml E 2broth medium with different carbon sources like glucose, fructose, sucrose, galactose, maltose and xylose and incubated at optimum temperature.

**Effect of nitrogen sources on PHB production:** The isolates were activated in E 2 broth medium with the best carbon source, and different 'N' sources like ammonium sulphate, ammonium chloride, ammonium nitrate, ammonium oxalate, ammonium heptamolybdate and yeast extract, with 1.0 g/l concentration and incubated at optimum temperature.

**Effect of different concentrations of the best N sources on PHB production:** The isolates were activated in E 2 broth medium having the best carbon source and different concentrations of the best N source i.e. 0.5, 1.0, 1.5 and 2.0 g/L and incubated at optimum temperature.

**Effect of temperature on PHB production:** The isolates were activated in E 2 broth medium with best carbon and nitrogen source and incubated at different temperatures viz. 25°C, 30°C and 37°C.

**Effect of pH on PHB production:** The isolates were activated in E 2 broth medium with best carbon and nitrogen source and pH of the medium was set at 6.0, 7.0, and 8.0 and incubated at optimum temperature.

**Effect of different C:N ratios on PHB production:** The isolates were activated in E 2 broth medium with different C:N ratios viz. 10:1, 15:1, 20;1 and 25:1 using the best C and N sources and incubated at optimum temperature.

**Determination of dry cell mass (DCM):** The isolates were activated in E 2 medium containing the optimized conditions and incubated for 48 hours. After incubation the cells were centrifuged to obtain pellet and washed with phosphate buffer and recentrifuged. Supernatant was thrown out and pellet was dried at 100°C for 24 hrs. The dried material was incubated at 60°C for 1 hr with 5% (v/v) sodium hypochlorite and centrifuged at 6000 rpm for 15 min. PHB was extracted using acetone alcohol method[26].

**Extraction by acetone-alcohol:** Cell mass (g/L) was obtained after 48 hrs of growth in E 2 agar mediumand centrifuged at 10,000 rpm for 10 min and lysed by sodium hypochlorite at 37°C for 1 hour. It was recentrifuged at 10,000 rpm for 10 min. This cell mass was washed with distilled water, followed by acetone: alcohol (1 : 1) and then by precipitating it in boiling chloroform (10 mL). The precipitate was allowed to evaporate at room temperature to obtain PHB in powder form[26].
% PHB production was calculated by the formula:

$$\%\ PHB = \frac{\text{Total weight of PHB}}{\text{Total weight of pellet}} \text{X100}$$

## Results and Discussion

**Quantitation and characterization:** The isolate KOL VII was quantified as the maximum producer with the absorbance 2.867 at 235 nm. The isolate then subjected to characterization, was found to be a gram positive rod which gave the colonial characteristics as off-white color, mucilaginous appearance, irregular shape, translucent, slightly raised colonies with serrate margins. No pigmentation was observed. It gave negative test for lactose fermentation and indole and $H_2S$ production whereas gave positive results for glucose and sucrose fermentation, catalase production, lipid hydrolysis, starch hydrolysis, gelatin liquefaction, nitrate reduction and acid production. The results showed the resemblance of the isolate towards the *Bacillus* species.

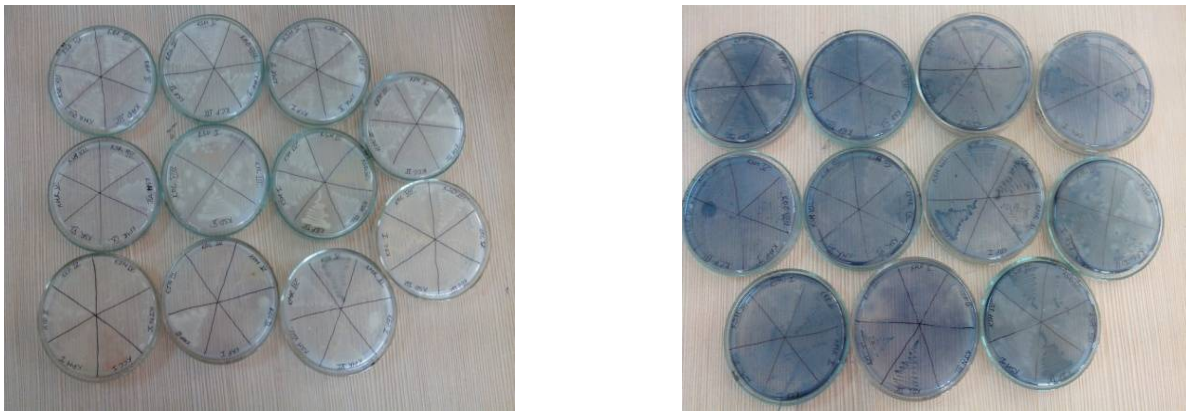

**Figure-1**
**Screening of the isolates by plate assay using Sudan Black B Dye**

**Table-1**
**Optimization results**

| Optimization factor | | Absorbance (235 nm) | | |
|---|---|---|---|---|
| | | **24 hours** | **48 hours** | **72 hours** |
| Carbon source | Glucose | 1.351 | 1.917 | 1.209 |
| | Galactose | 1.274 | 1.817 | 1.555 |
| | Sucrose | 1.726 | 3.913 | 3.068 |
| | Maltose | 0.982 | 1.465 | 2.163 |
| | Xylose | 0.983 | 1.668 | 1.653 |
| | Fructose | 0.541 | 1.917 | 1.608 |
| Nitrogen source | Ammonium sulphate | 0.219 | 1.715 | 1.263 |
| | Yeast extract | 1.160 | 2.914 | 2.142 |
| | Ammonium chloride | 0.804 | 1.724 | 1.625 |
| | Ammonium oxalate | 0.958 | 1.498 | 1.481 |
| | Ammonium nitrate | 0.500 | 1.016 | 0.892 |
| | Ammonium heptamolybdate | 0.451 | 1.644 | 1.323 |
| pH | 6.0 | 0.558 | 0.693 | 0.613 |
| | 7.0 | 0.387 | 0.882 | 0.572 |
| | 8.0 | 0.476 | 0.675 | 0.563 |
| Temperature | 25°C | 0.498 | 0.646 | 0.531 |
| | 30°C | 0.505 | 0.749 | 0.653 |
| | 37°C | 0.436 | 0.716 | 0.630 |
| Best N source | 0.5 g/L | 0.934 | 1.747 | 1.122 |
| | 1.0 g/L | 1.909 | 2.114 | 1.931 |
| | 1.5 g/L | 1.793 | 2.197 | 2.063 |
| | 2.0 g/L | 1.306 | 1.726 | 1.683 |
| C : N | 10 : 1 | 1.027 | 1.845 | 1.251 |
| | 15 : 1 | 1.813 | 2.767 | 2.659 |
| | 20 : 1 | 2.033 | 3.436 | 3.215 |
| | 25 : 1 | 0.606 | 2.323 | 1.595 |

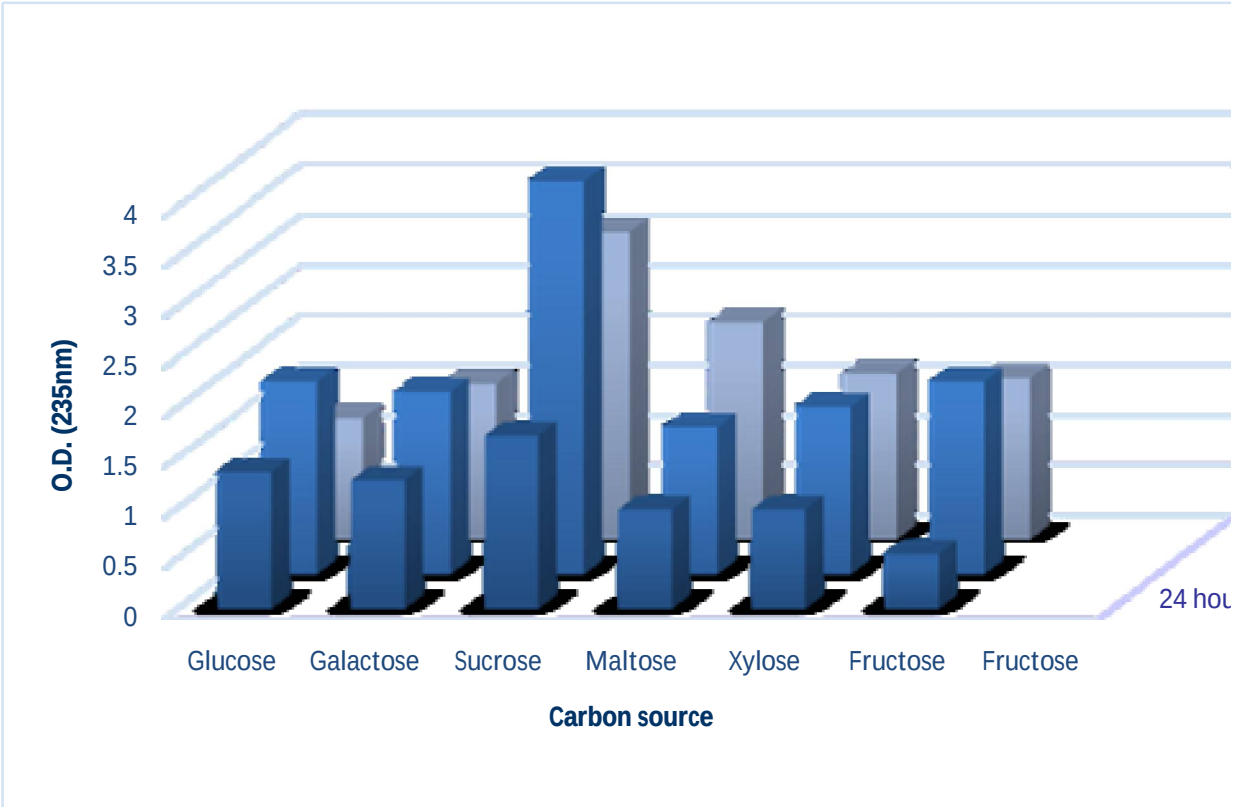


**Figure-2**
**Optimization result for different carbon sources**

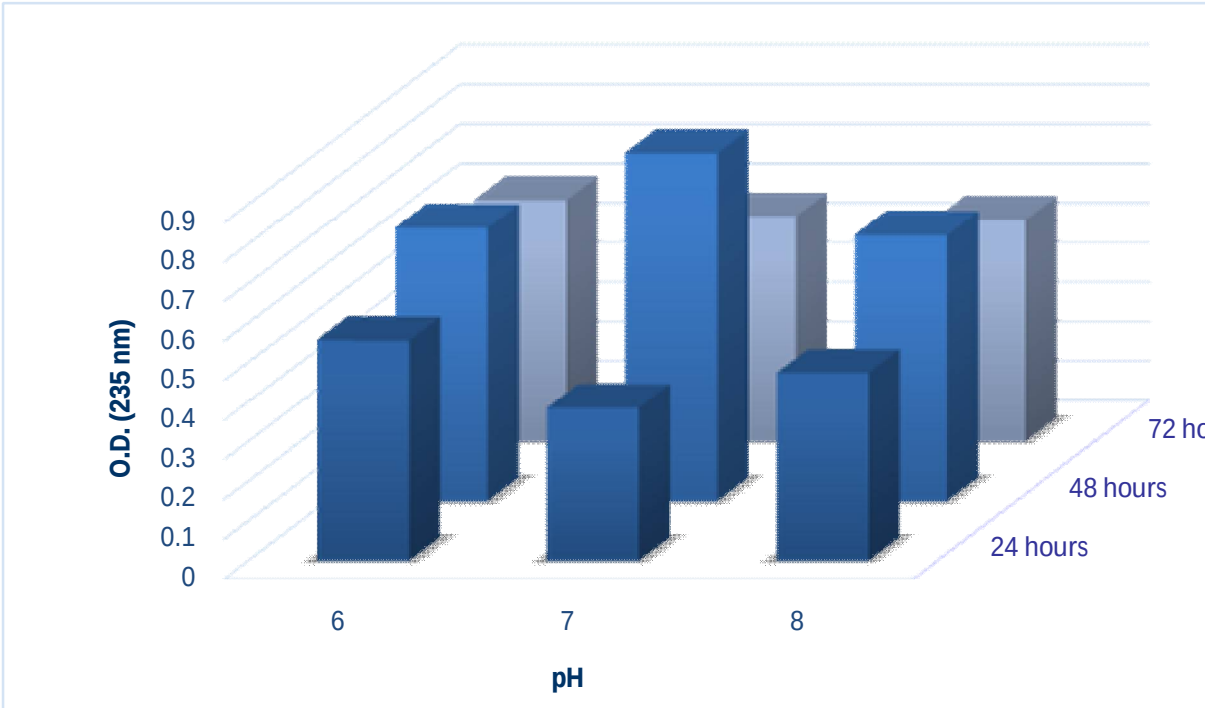


**Figure-3**
**Optimization result for different pH conditions**

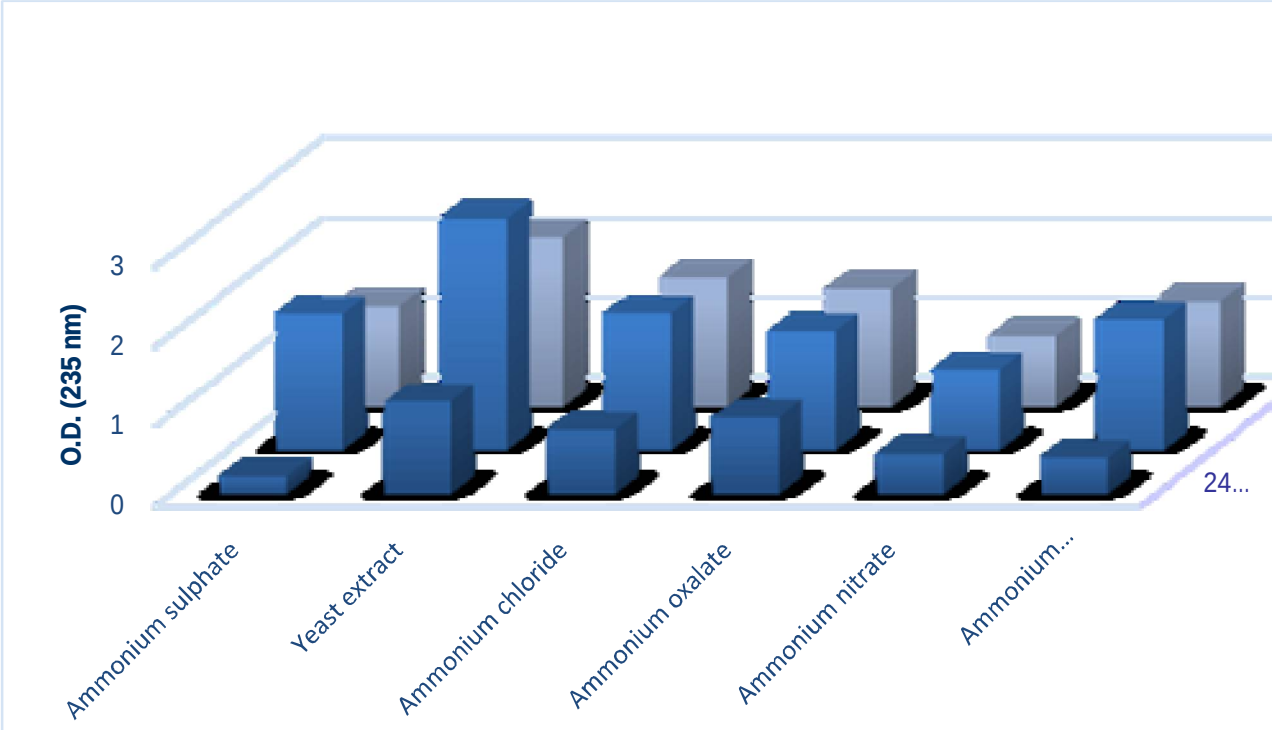


**Figure-4**
**Optimization result for different nitrogen sources**

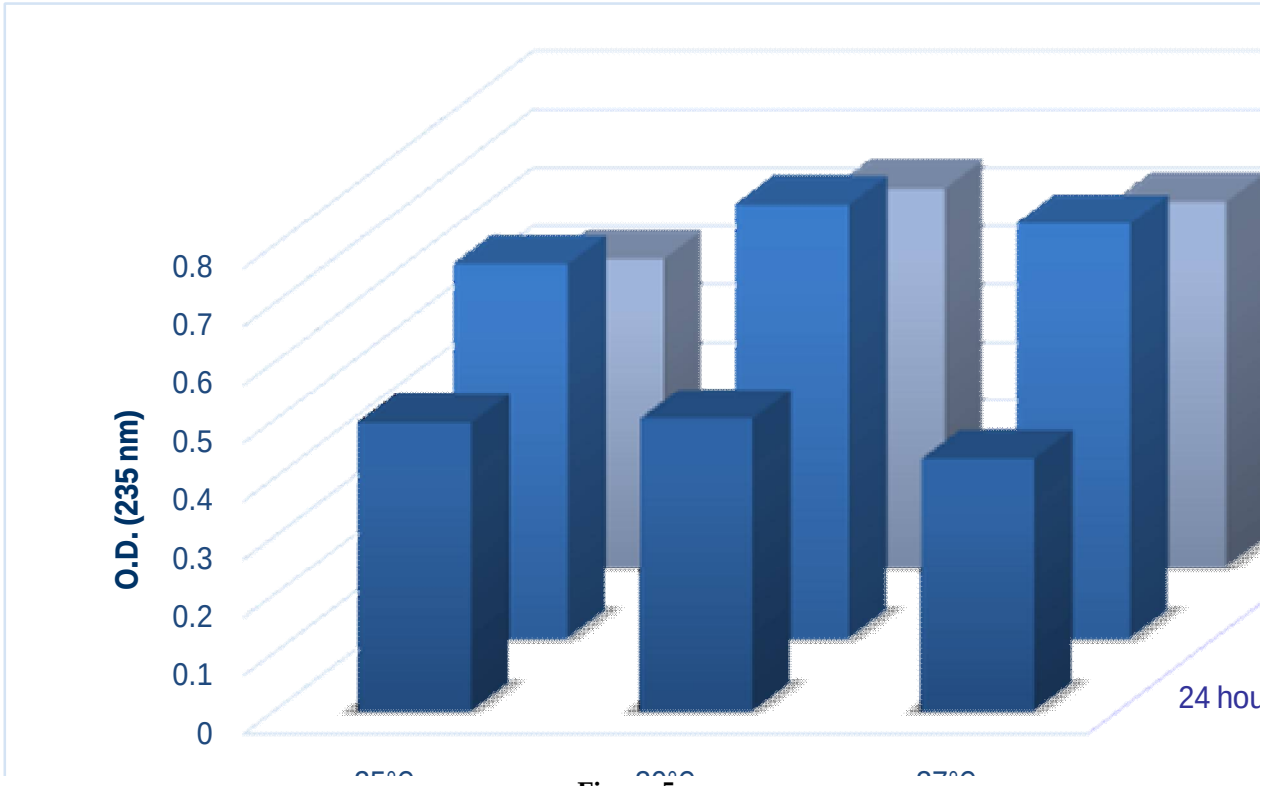


**Figure-5**
**Optimization result for different temperature conditions**

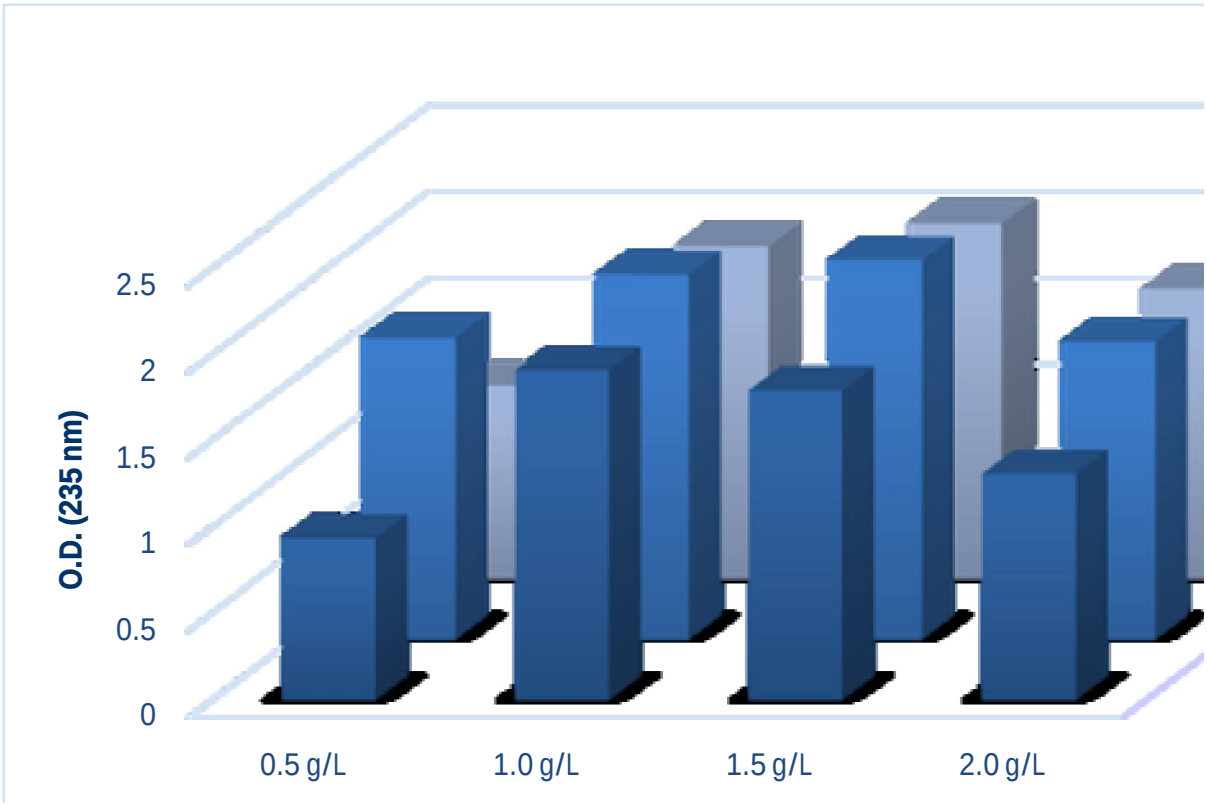


**Figure-6**
**Optimization for different concentrations of best nitrogen source**

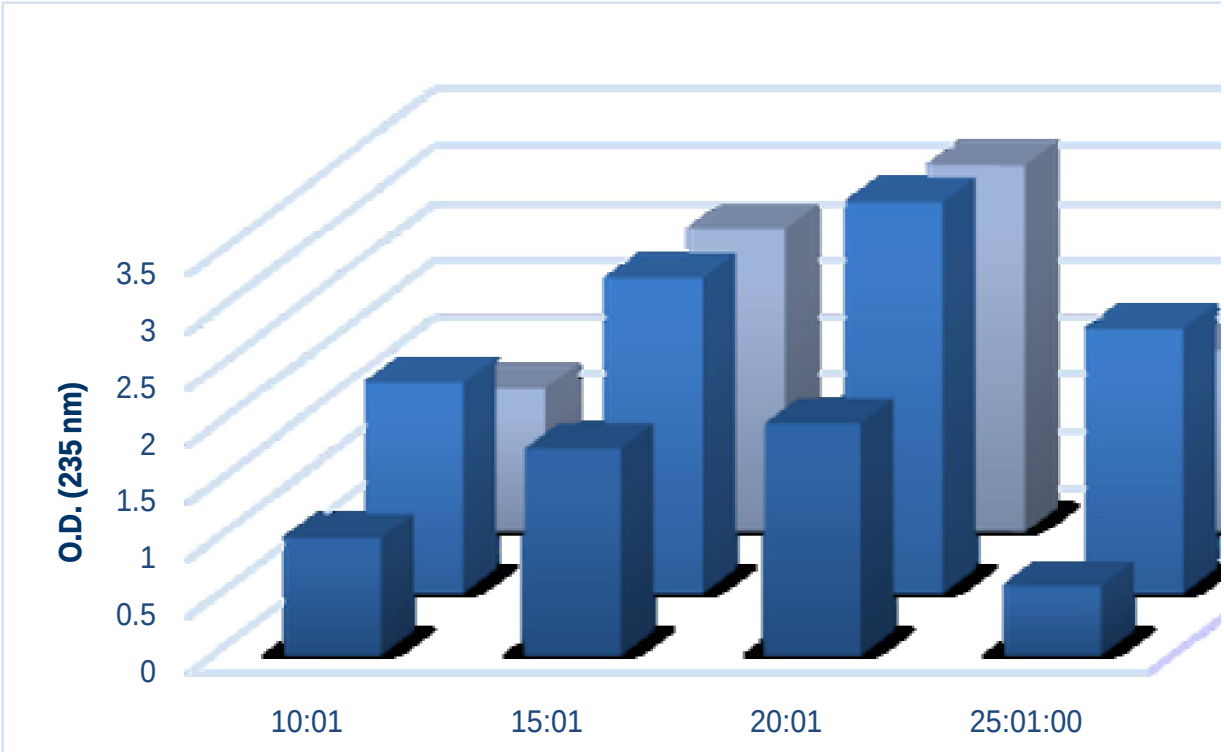


**Figure-7**
**Optimization result for different C:N ratios**

**Table-2**
**Dry cell mass (DCM) and % PHB content**

| **Dry Cell Mass** | |
|---|---|
| Weight of the crusible (W1) | 24.892 |
| Weight of the crusible with dry cell mass (DCM) (W2) | 24.743 |
| Dry cell mass (W) | 0.149 |
| **PHB content** | |
| Weight of the crusible (P1) | 24.675 |
| Weight of the crusible with PHB content (P2) | 24.746 |
| PHB content (P) | 0.071 |
| % PHB = P/W * 100 | 47.982% |

## Conclusion

Polyhydroxybutyrates (PHB) are bio-plastics that are produced by many microbial species under carbon rich and nutrient starvation conditions[18]. The organisms producing PHBs have been isolated, identified and the conditions of maximum production optimized[22,23]. Bioplastics have anextensivearray of agricultural, marine, industrial, environmental and medical applications which provides it its importance in the direction of investigation[1]. In present study, different organisms were isolated from various soil samples and were curtained for their knack to produce PHB in them. The positive isolates were quantified for their PHB production and the best isolate was further characterized and optimized for its ability to produce maximum amount of PHB. Considering future technical improvements and economies of scale, PHBs are more sustainable and eco-friendly alternative over petrochemical plastics[27]. Treatment of waste in this era is a challenging task which attracts a broad area of scope to this work. Fermented municipal wastewater solids can be efficiently used to accumulate high poly hydroxybutyrates (PHB) in the biomass, which may lead the whole scenario towards environmental friendly production. Unlike the petrochemical plastics, PHBs do not release ethene or any other hazardous gas in the atmosphere which generates a good scope in air pollution and municipal wastewater management by producing PHBs on the same. Advancement of proficient salvage methods is also an important aspect to lower the production cost of PHB. Since the carbon source books for 70 to 80% of total raw material cost, the PHB production cost can be ominously lowered using cheap carbon sources. The search for the favorable strains of PHA producers is an endless process and improvement of resource fulpolyhydroxybutyrate producing bacteria has become the need of the hour and engenders copious scopes for research in this field.

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