

*Original Research Article*

## Production of Poly- $\beta$ -Hydroxybutyrate (PHB) by bacteria isolated from rhizospheric soils

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### Abstract

*Polymeric materials like plastic and polyester have wide-spread use in today's industrial society because of their ease of processability and amenability in providing a large variety of cost-effective commodity. However, plastics and synthetic polymers are synthesized from nonrenewable resources like petrochemicals and persist in the environment long after intended use, resulting into problems of solid waste management and global environmental pollution. Therefore, there is a growing interest in developing biodegradable plastics such as polyhydroxybutyrate (PHB), which possess desirable physical and chemical properties similar to conventional synthetic plastics, and are environment friendly as well. In the present study, an attempt was therefore made to isolate efficient PHB producing bacteria from soil collected from the rhizospheric area of eight different crops. A total of 40 different types of bacteria were isolated, out of which 28 were found to be PHB positive, based on the viable colony staining method using Sudan Black B. All the 28 PHB positive isolates were characterized biochemically and subjected to quantitative estimation of PHB production, they were found to exhibit PHB yields in the range of 51.29 and 86.08 mg/ml. The culture medium and growth parameters for all the isolates were optimized for maximum PHB production. Glucose as the carbon and ammonium sulphate as the nitrogen source were found to be the best nutritional sources for maximum PHB production. Maintaining the C/N ratio as 20:1 using the best C and N source, pH of the medium at 7.0, and the temperature at 30°C were found to be optimum conditions for obtaining maximum PHB yield. A few (Sa7, Su4, De2, De1, Ch2, Ja1) PHB positive isolates were found to be quite efficient PHB producers, thus, exhibiting a potential for their utilization in commercial PHB production.*

**Key words:** Biodegradable plastic, PHB production, optimization, culture medium parameters.

### 1. Introduction

Petrochemical based plastics including polypropylene, polyethylene and polystyrene are almost exclusively made from the nonrenewable resource, viz., petroleum; which is also the main source of energy for today's world (Yu, 2000). In the western world, a large amount of fossil carbon is used by chemical industries and a part of this is used for production of plastics and polymers every year (Eggersdorfer *et al.*, 1992). The current nationwide dependence on fossil fuels for manufacturing plastics is around 270 million metric tonnes per year (Khardenavis *et al.*, 2007). If petroleum keeps on being consumed at this speed for various applications, it will be almost completely dried

out in the next 60-80 years (Khare and Deshmukh, 2006). Additionally, accumulation of these non-degradable plastics in the environment has resulted into a global menace. The increased cost of solid waste disposal as well as potential hazards from incineration of wastes such as dioxin emission, thus, make synthetic plastic waste management a problem (Ojuman *et al.*, 2004). Consequently, for the past two decades, there has been a growing public demand that the petroleum-based plastics should be replaced by certain environmentally acceptable biodegradable polymers, which are made from renewable resources, and do not lead to depletion of finite resources; resulting into an increasing scientific interest in the development and use of biodegradable polymers as an

ecologically useful alternative to plastics. One such category of biopolymers, polyhydroxyalkonates (PHA) are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a larger extent. Plastics produced from PHAs have been reported to be truly biodegradable in both aerobic and anaerobic environments (Page, 1995), unlike many of the “so-called” biodegradable plastics made synthetically.

Poly-beta hydroxybutyric acid (PHB) is the most popular and the best characterized polymer belonging to the class of polyhydroxyalkanoates. It is a kind of polyester synthesized by various microorganisms as energy reserve material under unfavorable conditions, *i.e.* during limitation of some essential nutrients or excess availability of carbon source (Lee, 1994); playing the same role in bacteria as fat in humans or starch in plants. Properties of pure PHB are comparable to commonly used bulk plastics, e.g. polypropylene. It is a unique natural biopolymer, which combines three exceptional features: (i) thermoplastic processability; (ii) 100% resistance to water and moisture; and (iii) 100% biodegradability (Hrabak, 1992). It could therefore be used for applications similar to those of common plastics and would fit well into new waste-management strategies as well. PHB has been detected to exist in various taxonomically different strains, many kinds of microbes such as *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Methylophil* are able to generate PHB up to 30 – 80% of their dry cell weight (Lafferty *et al.*, 1988). The rhizospheric soil layer associated with a large number of plants has been found to be colonized by numerous bacteria, many of which are potentially able to accumulate polyhydroxybutyrate as energy and carbon sources, thus, making it a good source for the isolation of PHB producers (Foster, 1985).

Although production of PHB can be realized through chemical synthesis, the production of biodegradable plastics on a large scale is limited because of the relative expense of the substrate and low polymer production. The higher production costs, especially raw material costs, make it difficult for PHB plastics to compete with conventional petroleum-based plastics in the commercial market place (Sangkharak and Prasertsan, 2008). Hence, alternative strategies for PHB production are being investigated. The success in the biodegradable plastic strategy largely depends on the isolation of potent PHB producing bacteria and optimizing culture medium parameters for maximum PHB biosynthesis. So, keeping these points in view, the present study was designed to isolate PHB producing bacteria from the rhizospheric area of eight different crops, and to optimize growth and culture conditions such as carbon source, nitrogen source, C/N ratio, pH, and incubation time and temperature for maximizing PHB production by them.

## 2. Materials and methods

### 2.1. Standard bacterial culture

A standard PHB positive bacterial strain, viz., *Cupriavidus necator* - MTCC-1472 (submitted as *Alcaligenes eutropha*), was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. This was used as the reference strain for comparison of the native isolates collected in the present study.

### 2.2. Collection of samples

For the isolation of PHB producing bacteria, soil samples were collected from the rhizospheric area of eight different crops (fenugreek, barley, mustard, chickpea, sugar beet, amaranth, Sesbania, and potato) commonly grown in India. The soil samples were collected, kept in plastic bags, marked with collection details and then stored at low temperature (40°C) till further use. For further processing, the collected samples were air dried at room temperature, and then gently crushed with the help of pestle and mortar for further analysis.

### 2.3. Bacterial isolation from collected soil samples

For the isolation of bacterial population, 10 gram of each soil sample collected from the rhizospheric area of different crops was suspended in 90 ml of sterile distilled water, shaken vigorously and serially diluted in sterile distilled water. Dilutions ranging from 10<sup>-5</sup> to 10<sup>-8</sup> were then plated on standard nutrient agar medium. After 48 hrs of incubation at 30°C, well-formed colonies were obtained on the plates. The total number of bacterial colony forming units (cfu) of each sample was enumerated, and the colonies were then studied for their morphological characteristics on the basis of their physical appearance (colour, size, shape and texture). Colonies showing remarkable differences in their morphology were selected and re-streaked on nutrient agar plates to obtain pure cultures.

### 2.4. Screening of isolates for PHB production using Sudan Black dye

All the representative pure isolates were screened for PHB production using the lipophilic stain Sudan Black B (Murray *et al.*, 1994) on agar plates, and under light microscope.

#### 2.4.1. Screening for PHB on solid agar

Individual bacterial isolates were streaked on nutrient agar plates (4-5 isolates on one plate), and the plates were incubated at 30°C for 24 hrs. Ethanolic solution

of 0.3% (w/v in 70% ethanol) Sudan Black B was spread over the colonies and the plates were kept undisturbed for 30 minutes. The plates were then destained by washing with ethanol (96%) to remove excess stain from the colonies. The colonies that retained their black colour after destaining were attributed as PHB producing strains (Mohamed *et al.*, 2012).

#### 2.4.2. Screening for PHB production under light microscope

For microscopic studies, smears of respective colonies were prepared on glass slides, heat fixed and stained with a 0.3% (w/v in 70% ethanol) solution of Sudan Black B for 10 min. The colonies were decolorized by immersing the slides in xylene, and were then counterstained with safranin (5% w/v in sterile distilled water) for 10 sec. Bacterial cells appearing black under the microscope were considered PHB producing strains while others were marked as negative (Legat *et al.*, 2010). All the positive isolates were assigned code numbers based on their source of isolation.

#### 2.5. Morphological and biochemical characterization of PHB positive isolates

The PHB positive isolates were grown on nutrient agar plates and their colony morphology was recorded. The morphological characteristics of the representative bacterial isolates (from each soil sample) showing differences in their physical appearances were recorded under four major headings, *viz.*, size, colour, shape, and texture. All these isolates were also studied under the microscope with respect to their cellular morphology and Gram staining properties (Gram, 1884). Biochemical characteristics of the isolates were studied following the standard microbiological methods described by Williams *et al.* (1994). Identification of the isolates was carried out on the basis of the results of morphological, cellular and the biochemical characteristics studied. Molecular characterization of the isolates is underway.

#### 2.6. PHB extraction and quantification

Polyhydroxybutyrate polymer was extracted and the amount of PHB produced was calculated from the standard curve prepared by using commercial poly- $\beta$ -hydroxybutyrate (Sigma-Aldrich) as per the method detailed by Law and Slepecky (1961). All the PHB positive bacterial isolates were raised in nutrient broth and the cell growth of each isolate containing the polymer was pelleted at 10,000 rpm at 4°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<sub>2</sub>SO<sub>4</sub> was added, which converts the polymer to crotonic acid, turning it into a brown coloured solution. The solution was cooled and absorbance was read at 235nm against a concentrated H<sub>2</sub>SO<sub>4</sub> blank on UV-VIS spectrophotometer (Nehra *et al.*, 2015). The quantity of PHB produced was determined by referring to the standard curve.

##### 2.6.1. Preparation of standard curve

Pure PHB (Sigma, USA) was used to prepare the standard curve of PHB. Two gram of PHB was dissolved in 10 ml of concentrated H<sub>2</sub>SO<sub>4</sub> and heated for 10 min. to convert PHB into crotonic acid, which gave 200 mg/ml of crotonic acid. From the above stock, working standard solutions were prepared by diluting it to obtain different concentrations ranging between 10 mg/ml to 150 mg/ml. Absorbance of all the dilutions was read at 235nm against a concentrated H<sub>2</sub>SO<sub>4</sub> blank on UV-VIS spectrophotometer, and the standard graph was made by plotting the various concentrations on the x-axis and the respective optical densities on the y-axis. The standard curve was used for estimation of PHB yield of the bacterial isolates.

##### 2.7. Optimization of culture medium parameters for maximum PHB production

Different factors *viz.*, carbon and nitrogen source, C/N ratio, pH, and incubation temperature play an important role in PHB production rate. Therefore, in order to determine the optimum conditions for maximum PHB production, the effect of all these parameters on PHB production by the PHB positive isolates was studied by varying all the conditions within a defined range.

##### 2.7.1. Optimization of different carbon sources

The effect of different carbon sources on PHB production was determined by raising the cultures of the PHB positive isolates in 100 ml of minimal salt medium (MSM) (Suresh Kumar *et al.*, 2004) supplemented with different carbon sources such as glucose, fructose, sucrose, maltose and arabinose at 2% concentration. Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 hrs. After incubation, PHB produced by the isolates was quantified

spectrophotometrically (as described earlier), and based on the yield, the best carbon source was determined.

#### 2.7.2. Nitrogen source optimization

The PHB positive isolates were inoculated in 100 ml of MSM broth containing the best carbon source and different nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate and yeast extract) at 1% concentration. After 48 h of incubation at 30°C, PHB yield was determined for all the isolates, and the best nitrogen source was selected on the basis of their yield.

#### 2.7.3. Optimization of Carbon to Nitrogen Ratio (C/N Ratio)

As PHB accumulation has been found to be enhanced if the bacterial cells are cultivated in the presence of an excess carbon and limited nitrogen sources (Reddy *et al.*, 2009), therefore, in addition to the determination of the best C and N sources, the effect of different C:N ratios on PHB production was also determined. For this, cultures were inoculated in MSM supplemented with different ratios of concentrations of the best C and N source (C/N ratio as 10:1, 15:1, 20:1 and 25:1). Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 h. After incubation, PHB yield was quantified spectrophotometrically, and based on the yields the best C/N ratio was determined.

#### 2.7.4. Effect of pH on PHB production

For pH optimization, cultures of the PHB positive isolates were raised in MSM supplemented with the best C and N source having different pH, viz., 6.0, 7.0 and 8.0. Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 hrs. After incubation, PHB yield was quantified spectrophotometrically, and the pH exhibiting maximum yield was determined.

#### 2.7.5. Effect of temperature on PHB production

The effect of different incubation temperatures on PHB production was determined by inoculating the cultures in MSM supplemented with the best C and N source and then incubating at different temperatures viz., 25°C, 30°C, 35°C, 40°C, and 45°C. After 48 h of incubation at respective temperatures, PHB yield was quantified spectrophotometrically; based on the yields the optimum temperature for maximum PHB production was determined.

### 3. Results and discussion

#### 3.1. Isolation and screening of PHB producing bacteria

Soil samples were collected from the rhizospheric region of eight different crops commonly grown in India (listed in table 1). Total bacterial population was enumerated by making serial dilutions of each soil sample and plating appropriate dilutions on nutrient agar medium. Based upon the morphological differences in their colony characteristics (size, shape, colour and texture), a total of 40 representative bacterial colonies (four each from the rhizospheric area of Fenugreek and barley; eight each from mustard and sugarbeet rhizosphere; five from chickpea rhizosphere; six from amaranthus rhizosphere; two from Sesbania rhizosphere; and three from the potato rhizospheric soil) were picked, purified and maintained as pure cultures for further screening using Sudan Black dye.

The screening was done by staining the isolates with Sudan Black B on petri plates as well as under the microscope. Sudan Black dye has been used as a screening measure for PHB production by several workers (Poirier *et al.*, 1995; Suresh Kumar *et al.*, 2004; Singh *et al.*, 2011; Soam *et al.*, 2012;). It was observed that out of 40 isolates, as many as 28 isolates (three each from the rhizospheric area of fenugreek, barley, chickpea and amaranth; six each from mustard and sugarbeet rhizosphere; and two each from Sesbania and potato rhizospheric soil) were found to accumulate PHB, exhibiting blue/ black colour, both under the microscope and on the plates upon staining with Sudan Black (Fig. 1). All the positive isolates were assigned code numbers based on their source of isolation (Table 1).

#### 3.2. Characterization of PHB positive isolates and quantification of their PHB production

All the 28 Sudan Black B positive isolates were subjected to morphological, microscopic and standard biochemical characterization. Morphological characteristics of all the isolates were studied in terms of their colour, shape, texture and size. The isolates exhibited high variability with regard to all the characteristics: being white/ off-white/ yellowish-orange in colour; having a round or irregular shape with a glossy/ smooth/ dry texture; and the size varying between small to large. Gram staining of the isolates revealed that the majority of the isolates (82%) were Gram negative in nature, there being only five Gram positives among a total of 28 isolates (Table 2). In literature, both Gram negative and Gram positive bacteria have been reported to accumulate PHB (Preiss, 1989). Identification of the isolates was carried out by studying the results of standard biochemical tests as per the details given in the Bergey's Manual of Systematic Bacteriology (Williams *et al.*, 1994). On a preliminary basis, the isolates have been found to

belong to five genera, viz., *Escherichia*, *Enterobacter*, *Bacillus*, *Staphylococcus* and *Pseudomonas*. Molecular identification of the isolates is underway.

### 3.3. Quantification of PHB production

The PHB yield of all the 28 isolates and the standard strain *Cupriavidus necator* (MTCC 1472) was determined using the method described by Law and Slepecky (1961), results of which have been detailed in Table 2.

The yield of the isolates was found to vary between 51.29 mg/ml (Me3 isolate) to 86.08 mg/ml (Sa7 isolate), however, the PHB yield of the reference strain MTCC 1472 being higher (144.23 mg/ml) than the isolates. Among the 28 PHB positive isolates obtained from the rhizospheric area of different crops, highest PHB producers were observed to belong to the *Sesbania* rhizosphere isolates (showing an average of 83.94 mg/ml of PHB), followed closely by the isolates belonging to the chickpea rhizosphere (with an average PHB yield of 77.49 mg/ml). Bacterial isolates from the rhizospheres of barley, mustard, sugarbeet and fenugreek exhibited almost a similar pattern, with a few isolates showing a high PHB yield, whereas the others with a lower yield; having an average PHB yield of 72.66, 71.92, 70.83 and 70.00, respectively. The lowest PHB yield was exhibited by the isolates belonging to the potato and amaranth rhizosphere. Although the PHB yield was found to be different for the isolates belonging to different crop rhizospheres; but further studies with a wider range of initial sampling data are required before deriving a correlation between the PHB yield of an isolate and the effect of rhizospheric soil atmospheres of different crops. Plans for these studies are underway.

### 3.4. Optimization of culture medium constituents and growth conditions for maximum PHB production

PHB accumulation by different bacteria has been reported to be affected by their nutritional, growth and physical factors such as the C-source, N-source, C/N ratio, pH and temperature (Reddy et al., 2009). Therefore, all these parameters were optimized for maximum PHB production by all the 28 isolates.

#### 3.4.1. Effect of different carbon sources on PHB yield

All the PHB positive isolates were grown in the presence of five different carbon sources, viz., glucose, fructose, sucrose, maltose and arabinose, and the PHB yield in the presence of each of these different C-sources was recorded (Fig. 2). Amongst the different carbon sources tested to evaluate their effect on PHB yield, glucose was found to be the best carbon source.

It yielded a mean PHB yield of 91.09 mg/ml. This was followed by fructose with a mean PHB yield of 85.42 mg/ml; further followed by sucrose and maltose, which showed almost a similar pattern of PHB accumulation. Least PHB production was observed when the medium was supplemented with arabinose as the C-source. In previous similar studies, PHB accumulation has been studied with different sugars by different scientists; however, high PHB yields have been reported upon utilization of sugars such as fructose (Khanna and Srivastava, 2005) and maltose (Soam et al., 2012). It has been reported earlier (Khanna and Srivastava, 2005) that monosaccharides such as glucose and fructose are readily utilized by bacteria and, hence support growth and subsequently PHB production. On the other hand, complex molecules like starch and lactose are not easily utilized for effective PHB production. As the complexity of the carbon sources increases, PHB yield has been found to decrease (Joshi and Jayaswal, 2010). Results of the present study are also in consonance with these reports as higher PHB accumulation was obtained with glucose and fructose as compared to the more complex sugars tested.

The interaction effects of the isolate and carbon sources were also found to be significant. Amongst the different PHB isolates De1 was significantly superior when compared to all other isolates. It recorded the highest PHB yield of 116.44mg/ml on glucose as the carbon source (2%), and was found to produce significantly higher yield on fructose (110.77 mg/ml) also. De1 was followed by Co4 and Po3, wherein again the PHB accumulation was found to be higher with glucose and fructose than with other sugars.

#### 3.4.2. Effect of different nitrogen sources on PHB yield

To study the effect of nitrogen and to select the best N-source for maximum PHB production, four different nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate and yeast extract) were included in the MSM medium along with the best C-source (glucose, 2%), and the results are depicted in Fig. 3. Amongst different N sources, ammonium sulphate was found to be the best N source. It produced a mean PHB of 91.48 mg/ml. The next promising N sources were ammonium chloride with 86.52 mg/ml and ammonium nitrate with 81.44 mg/ml PHB yields. Yeast extract was found to be least supporter of PHB production. The interaction effects of isolates and N sources were also found to be significant. Amongst the isolates, De1 was again found to be a significantly high PHB producer compared to all other isolates, producing a mean PHB of 105.49 mg/ml. De1 on ammonium sulphate produced the highest PHB yield of 112.53 mg/ml, much higher than its yield in nutrient broth (82.11 mg/ml). The results in

the present study are in agreement with several earlier reports. Khanna and Srivastava (2005), working with *R. eutropha* reported highest PHB production on MSM medium supplemented with ammonium sulphate. Similarly, Mulchandani *et al.* (1989), and Raje and Srivastava (1998), working on the accumulation of PHB by *A. eutrophus* obtained highest PHB yield in ammonium sulphate followed by ammonium chloride. Ammonium sulphate being a simple nitrogen source is probably more readily available than the other complex nitrogen sources. However, in contrast to these results, a few studies have also reported high PHB production with ammonium nitrate (Soam *et al.*, 2012).

### 3.4.3. Effect of relative concentrations of carbon and nitrogen sources on PHB production

Different C:N ratios (10:1, 15:1, 20:1 and 25:1) were maintained using the best carbon (glucose) and the best nitrogen source (ammonium sulphate) in the minimal salt medium and their effect on PHB production was studied (Fig. 4). Amongst the different C/N ratios tested, 20:1 was found to be the best C:N ratio, supporting the highest PHB production (with an average of 79.29 mg/ml). All the isolates showed an increase in PHB accumulation with an increase in C:N ratio, but only upto 20:1, beyond which a decrease was observed. This decrease may be attributed to the phenomenon of substrate inhibition. Similar observations have been made by Belal, 2013, and Panigrahi and Badveli, 2013. Amongst the different isolates, highest PHB production at C/N ratio of 20:1 was observed for De1 (99.23 mg/ml), followed by that of De2 (95.52 mg/ml).

### 3.4.4. Effect of pH on PHB production

Different pH conditions (pH 6.0, 7.0 and 8.0) were maintained in the media prepared using the best carbon and nitrogen (glucose and ammonium sulphate) sources and their effect on PHB production was evaluated (Fig. 5). Out of the different pH of media tested, pH 7.0 was found to be best for maximum PHB production by all the isolates. At pH 6.0, all the isolates were found to produce very low yields showing that pH 6.0 was not much suitable for PHB accumulation. However, at pH 8.0, although the PHB yield was not too low, but still it was low as compared to the yield at pH 7.0, thus, establishing that pH 7.0 was the optimum pH for PHB accumulation. These results are in accordance with the results obtained by Grothe *et al.* (1999) who have reported that pH values ranging from 6.0 to 7.5 are optimum for PHB production. In the present study, pH 7.0 resulted in a mean PHB production of 76.17mg/ml, while the average PHB production was 67.66mg/ml at pH 8.0,

and 49.62mg/ml at pH 6.0. De2 was the best isolate with mean PHB production of 74.07mg/ml. At pH 7.0, the highest PHB of 95.21mg/ml was produced by De2 which was significantly higher than all the isolates.

### 3.4.5. Effect of different incubation temperatures on PHB yield

For studying the effect of incubation temperatures on PHB production, different temperatures (25<sup>o</sup>C, 30<sup>o</sup>C, 35<sup>o</sup>C, 40<sup>o</sup>C and 45<sup>o</sup>C) were maintained during incubation of the isolates in a medium prepared using the best carbon and nitrogen (glucose and ammonium sulphate) sources. Data are presented in Fig.6. The incubation temperature of 30<sup>o</sup>C was found to be optimum for maximum PHB production by all the isolates. It yielded a mean PHB of 74.25mg/ml. This was followed by the incubation temperature of 35<sup>o</sup>C with a mean of 68.70 mg/ml PHB yield. The isolate Ja1 was found to produce a PHB yield of 85.52mg/ml at 30<sup>o</sup>C. It was found to produce significantly high yields at 35<sup>o</sup>C temperature also. However, at temperatures below 30<sup>o</sup>C and beyond 35<sup>o</sup>C, the PHB yield dropped significantly, suggesting that very low or very high temperatures did not support PHB production. Similar results have been obtained by Grothe *et al.* (1999), wherein they have concluded that 33<sup>o</sup>C temperature is optimum for PHB synthesis under fermentation conditions. The data from this study also confirms that although 30<sup>o</sup>C was the optimum temperature for PHB production, but high PHB accumulation was also observed at 35<sup>o</sup>C.

## 4. Conclusion

The major objective of the present study was to isolate effective polyhydroxybutyrate producing strains and to optimize their culture conditions so as to obtain the maximum PHB yield. According to the results of the present study, the optimum culture conditions for maximum PHB production by a wide range of soil bacteria include supplementation of the culture medium with glucose as C-source, ammonium sulphate as the nitrogen source with the C:N ratio being maintained as 20:1, pH as 7.0 and incubation temperature at 30<sup>o</sup>C. Two promising isolates, viz., De1 and De2 were found to accumulate a high level of PHB at the optimized culture conditions; thus, showing a potential for their exploitation in industrial PHB production. The present study has thus provided useful data about the optimized conditions for PHB production that can be utilized for industrial production of PHB, a fast emerging alternative of non biodegradable plastics.

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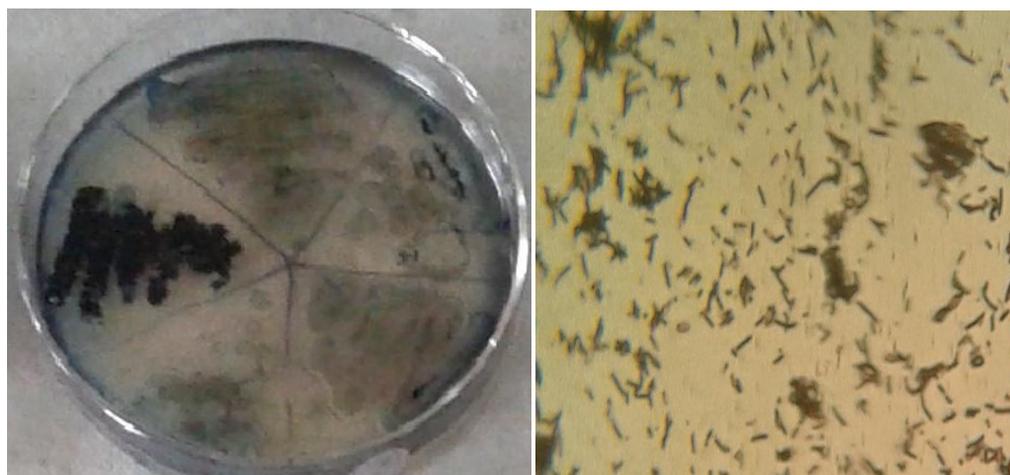
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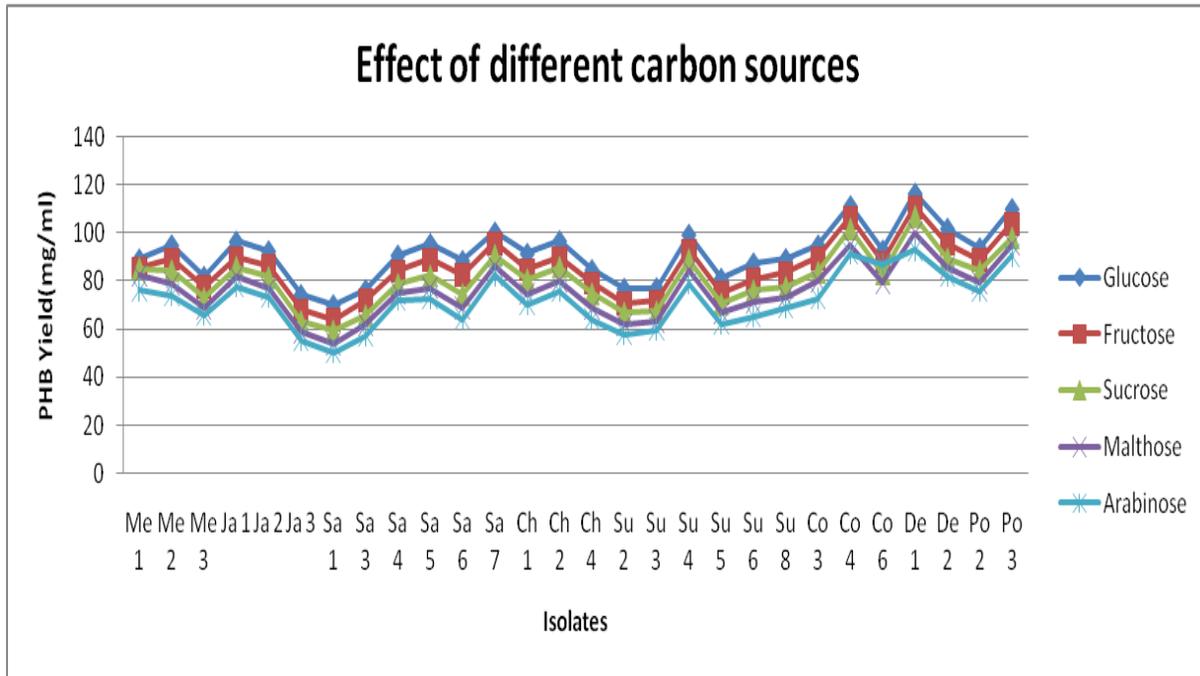
**Table 1:** Isolation of PHB positive bacteria from soil samples from the rhizospheric area of different crops.

| Sr. No. | Soil Sample (Crop rhizosphere)<br>Common name<br>(Scientific Name) | Total no. of different types of isolates | No. of PHB positive isolates |
|---------|--|--|------------------------------|
| 1.      | Fenugreek/ Methi<br><i>Trigonella foenum graecum</i>               | 4  | 3 (Me1 to Me3)               |
| 2.      | Barley/ Jau<br><i>Hordeum vulgare</i>                              | 4  | 3 (Ja1 to Ja3)               |
| 3.      | Mustard/ Sarson<br><i>Brassica nigra</i>                           | 8  | 6 (Sa1, Sa3 to Sa7)          |
| 4.      | Chickpea/ Channa<br><i>Cicer arietinum</i>                         | 5  | 3 (Ch1, Ch2, Ch4)            |
| 5.      | Sugar beet<br><i>Beta vulgaris</i>                                 | 8  | 6 (Su2 to Su6, Su8)          |
| 6.      | Amaranth/ Chola<br><i>Amaranthus polygamous</i>                    | 6  | 3 (Co3, Co4, Co6)            |
| 7.      | Dhaincha<br><i>Sesbania bispinosa</i>                              | 2  | 2 (De1, De2)                 |
| 8.      | Potato/ Aaloo<br><i>Solanum tuberosum</i>                          | 3  | 2 (Po2, Po3)                 |
|         | <b>TOTAL</b>   | <b>40</b>                                | <b>28</b>                    |

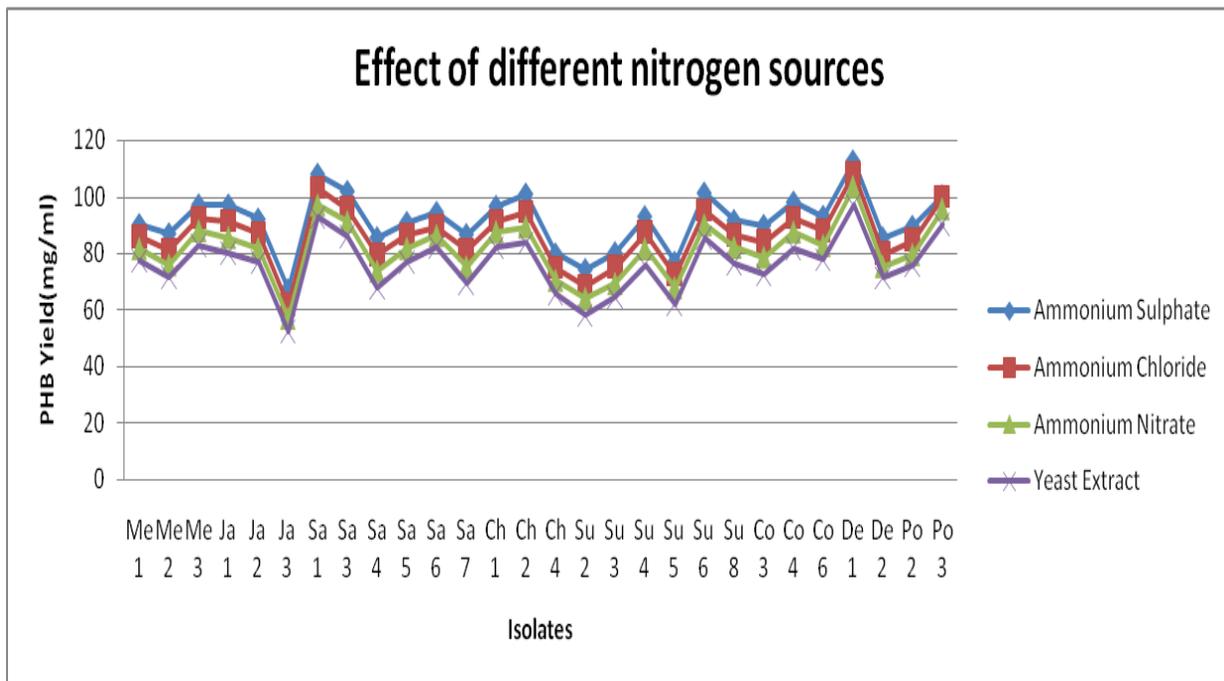
**Table 2:** Gram staining properties and PHB production by Sudan Black B positive isolates.

| Sr. No. | Crop Rhizosphere  | PHB positive Isolate | Gram Staining | PHB Yield (mg/ml) | Average PHB Yield (mg/ml) |
|---------|---|----------------------|---------------|-------------------|---------------------------|
| 1.      | Methi (Fenugreek)   | Me1                  | -ve           | 79.18             | 70.00                     |
| 2.      |   | Me2                  | -ve           | 79.54             |                           |
| 3.      |   | Me3                  | -ve           | 51.29             |                           |
| 4.      | Jau (Barley)  | Ja1                  | -ve           | 81.96             | 72.66                     |
| 5.      |   | Ja2                  | +ve           | 77.06             |                           |
| 6.      |   | Ja3                  | -ve           | 58.97             |                           |
| 7.      | Sarson (Mustard)  | Sa1                  | -ve           | 65.72             | 71.92                     |
| 8.      |   | Sa3                  | -ve           | 59.12             |                           |
| 9.      |   | Sa4                  | -ve           | 74.95             |                           |
| 10.     |   | Sa5                  | -ve           | 77.27             |                           |
| 11.     |   | Sa6                  | +ve           | 68.40             |                           |
| 12.     |   | Sa7                  | -ve           | 86.08             |                           |
| 13.     | Channa (Chickpea)   | Ch1                  | -ve           | 81.08             | 77.49                     |
| 14.     |   | Ch2                  | -ve           | 82.78             |                           |
| 15.     |   | Ch4                  | -ve           | 68.61             |                           |
| 16.     | Sugarbeet ( <i>Beta</i> )                                 | Su2                  | -ve           | 61.75             | 70.83                     |
| 17.     |   | Su3                  | -ve           | 63.25             |                           |
| 18.     |   | Su4                  | -ve           | 85.82             |                           |
| 19.     |   | Su5                  | -ve           | 66.86             |                           |
| 20.     |   | Su6                  | +ve           | 73.81             |                           |
| 21.     |   | Su8                  | -ve           | 73.51             |                           |
| 22.     | Cholai ( <i>Amaranth</i> )                                | Co3                  | -ve           | 79.95             | 69.23                     |
| 23.     |   | Co4                  | -ve           | 48.56             |                           |
| 24.     |   | Co6                  | -ve           | 79.18             |                           |
| 25.     | Dhaincha ( <i>Sesbania</i> )                              | De1                  | +ve           | 82.11             | 83.94                     |
| 26.     |   | De2                  | +ve           | 85.77             |                           |
| 27.     | Potato ( <i>Solanum</i> )                                 | Po2                  | -ve           | 79.43             | 69.27                     |
| 28.     |   | Po3                  | -ve           | 59.12             |                           |
| 29.     | Standard Strain<br><i>Cupriavidus necator</i> (MTCC 1472) |                      | -ve           | 144.23            |                           |

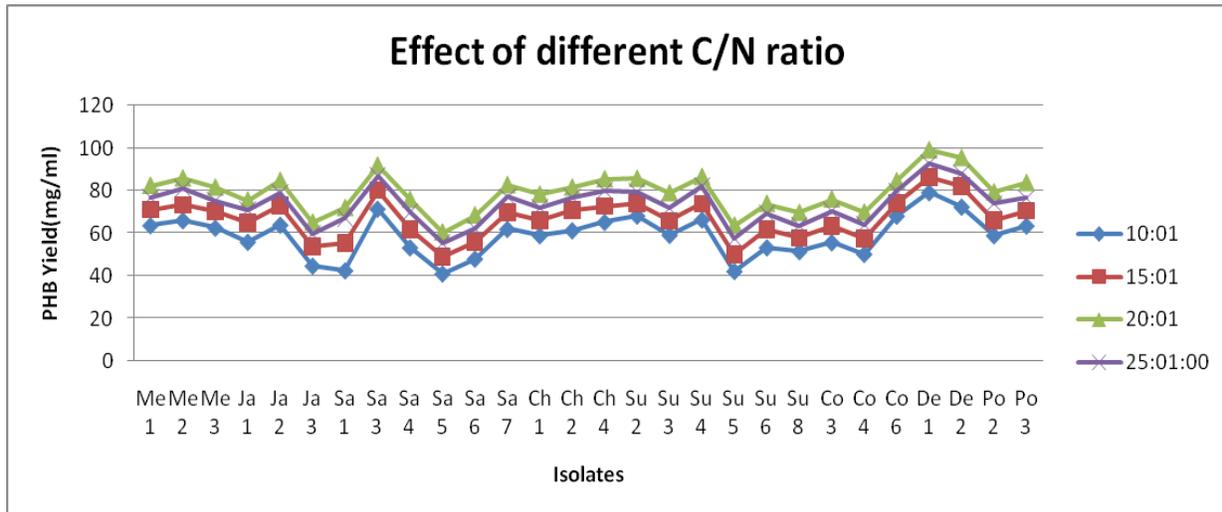
**Fig. 1:** Selected blue black colored colonies after Sudan Black B staining.



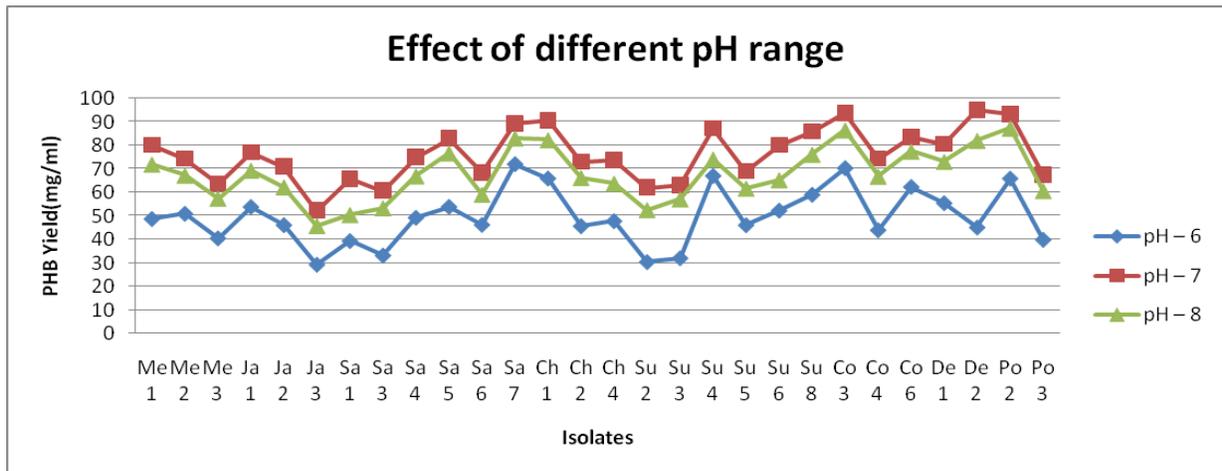
**Fig. 2 :** Effect of different carbon sources on PHB production by different isolates.



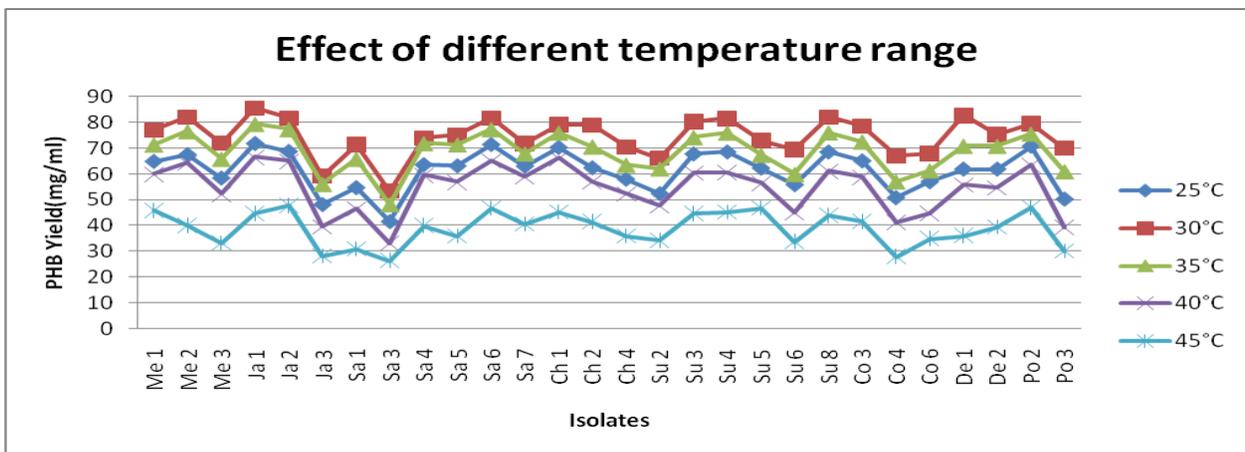
**Fig. 3:** Effect of different nitrogen sources on PHB production by different isolates



**Fig. 4:** Effect of different C/N ratios on PHB production by different isolates



**Fig. 5:** Effect of pH alteration on PHB production by different isolates



**Fig. 6:** Effect of incubation temperature on PHB production by different isolates