

Original Research Article

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## Production of Short Chain Length Polyhydroxyalkanoates by *Bacillus megaterium* PHB29 from Starch Feed Stock

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### ABSTRACT

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Polyhydroxyalkanoates (PHAs) are accumulated in bacteria as an energy reserve and have attracted great scientific interest as a biodegradable, biocompatible alternative to conventional plastics. The study is focused on polyhydroxybutyrate (PHB) accumulation by an environmental bacterial isolate *Bacillus megaterium* PHB29 using starch as carbon feedstock. PHB accumulation in the isolate was visualized by staining techniques using Sudan Black B and Nile Red dyes. The biomass production as well as PHA accumulation in PHB29 was tested with starch as carbon source at different temperatures and found yielding 3.07 g/L cell dry mass with 73.46% (w/w) PHA accumulation at an optimum temperature 34°C. The extracted polymer was subjected to <sup>1</sup>H NMR analysis and the spectrum showed signals for a methine group (-CH-), a methylene group (-CH<sub>2</sub>-) and a methyl group (-CH<sub>3</sub>) proving the polymer as poly-3-hydroxybutyrate (PHB). The ratio of starch to PHB conversion of this isolate was found to be better than other reported strains of *Bacillus megaterium* as well as other species among the genus *Bacillus*.

### Introduction

Polyhydroxyalkanoates (PHAs) are polyoxoesters of R-hydroxyalkanoic acid monomers accumulated in bacterial cytoplasm as granules when carbon sources are available in excess and other nutrients such as nitrogen and phosphorus are limited in their surroundings (Dawes and Senior, 1972; Lee *et al.*, 1999). They act as carbon-energy reserves and also as electron sinks, which enhance the fitness of the bacteria and help in maintaining the redox balance

(López *et al.*, 1995; Madison and Huisman 1999; Ruiz *et al.*, 2006). These materials are biocompatible, non-toxic and considered as alternatives for conventional petroleum derived plastics (Chen, 2010). Depending on the bacterial strains, growth substrates and incubation conditions, the monomer composition of the accumulated polymer may vary and as a result, the properties of PHAs range from thermoplastics to elastomers (Braunegg *et*

*al.*, 1998; Keshavarz and Roy, 2010). The main attraction of PHAs is that, they are entirely degraded to carbon dioxide and water through natural microbial process. Consequently, their production, use and degradation never affect the environment adversely (Braunegg *et al.*, 1998).

Most PHAs have been produced by prokaryotic microorganisms, including bacteria, cyanobacteria and archaea (Panda *et al.*, 2005; Quillaguaman *et al.*, 2005; Leong *et al.*, 2014). Bacteria coming under the genera *Bacillus* are well known for their ability to accumulate poly-3-hydroxybutyrate (PHB) which is the most common and simplest form of PHA with 3-hydroxybutyrate (C<sub>4</sub>) monomers. This class of short-chain-length (SCL) PHA is the first discovered and the most extensively studied biopolymer (Łabużek and Radecka 2001; Shamala *et al.*, 2003; Singh *et al.*, 2009; Mizuno *et al.*, 2010).

Even if polyhydroxyalkanoates have ideal properties as a substitute to environment polluting petroleum derived plastics, their applicability is limited due to their high cost of production. The cost of raw materials contributes a significant share to the manufacturing cost of PHA. In this scenario, researches have been focusing on reducing the production cost by replacing the expensive substrates with renewable cheap raw materials (Du *et al.*, 2012). For industrial production of PHAs, starch may be a suitable cost effective alternative carbon source as it can be generated from various agricultural wastes (Chien and Ho 2008).

In this study, we used a starch utilizing bacteria isolated from the environment for analyzing its PHA accumulating property in a medium containing starch as carbon feed stock and observed simultaneous

saccharification and fermentation with high polymer yield.

## **Materials and Methods**

### **Bacterial strain**

The bacterium used in this study, *Bacillus megaterium* PHB29 was isolated from forest soil collected from Western Ghats region, Thiruvananthapuram District and was previously reported for its L-asparaginase production (Arjun *et al.*, 2015).

### **Visualization of PHA accumulation**

**Sudan Black B staining:** 1 µL of the overnight bacterial culture was spotted on half strength nutrient agar (Himedia Laboratories Pvt. Ltd, Mumbai, India) plate supplemented with 2% glucose. After 48 h of incubation at room temperature, the plate was flooded with 0.05% solution of Sudan Black B in ethanol and kept undisturbed for 30 min. The excess stain was washed out by sterile saline and observed the change in colony colour (Liu *et al.*, 1998).

10 µL of 48 h old bacterial culture grown in half strength nutrient broth (Himedia Laboratories) supplemented with 2% glucose was made into a smear on a microscopic slide and stained with Sudan Black B (Murray *et al.*, 1994). The smear was observed under 100X oil immersion objective lens of light microscope to visualize the polymer granules.

**Nile Red staining:** Cells from 1 mL of bacterial culture were harvested by centrifugation at 5000xg, for 3 min, washed with 0.1 M phosphate buffered saline (PBS) and fixed in 20% formaldehyde solution. 1 mL of the cell suspension was mixed with 100 µL of Nile red solution (1 µg/mL in

acetone) for 10 min. 10  $\mu$ L of bacterial cell suspension was taken on a glass slide and was covered by a cover slip (Greenspan *et al.*, 1985; Jendrossek *et al.*, 2007). The cells were imaged on a Nikon A1R-Si laser scanning confocal spectral microscope with 50X magnification at an excitation of 561 nm.

### **PHA production from starch**

The isolate was inoculated in 1000 mL basal medium (1.5 g of peptone, 1.5 g of yeast extract, 1 g of  $\text{Na}_2\text{HPO}_4$  and 0.2 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per liter) with 20 g/L of soluble starch (Himedia Laboratories) at pH 7.2 and incubated at different temperature conditions (3°C intervals between 28°C and 40°C) for 48 h. The experiments were done in triplicate and the cells harvested were washed with sterile normal saline. The biomass obtained was lyophilized and measured the cell dry mass (CDM). The polymer was extracted from dried biomass according to Shi *et al.*, (1997) with slight modification. The cell mass was dispersed in 20 mL of sodium hypochlorite solution (available chlorine 5% w/v) and incubated at 45°C for 1h. The lysate was pelleted at 8000xg for 2 min and washed successively with 20 mL of sterile distilled water, acetone and absolute ethanol. The polymer obtained was dissolved in 20 mL of boiling chloroform and transferred to a clean glass Petri plate to evaporate the solvent. The polymer film obtained was weighed and estimated the yield in percentage (w/w).

### **Identification of the polymer**

The proton Nuclear Magnetic Resonance Spectroscopy ( $^1\text{H}$  NMR) of the polymer was done after suspending the polymer in high purity deuteriochloroform ( $\text{CDCl}_3$ ).  $^1\text{H}$  NMR spectra of the polymer were recorded at 500 MHz and magnetic field strength of

11.7 T in BrukerAvance<sup>II</sup> 500 NMR spectrometer (Bruker Corporation, Massachusetts, USA).

## **Results and Discussion**

### **Visualization of PHA accumulation**

PHB29 bacterial colony appeared dark blue in colour and the *Escherichia coli* (negative control) colony remained colourless (Fig. 1A). Sudan Black B is a lipophilic dye which binds with polyesters accumulated inside the cells resulting in the dark blue stained colony morphology and *E. coli* colony remained colourless as it is not a natural PHA producer. The culture smear, when stained with Sudan Black B, the bacterial cells showed dark blue coloured granules in the cytoplasm, almost filled in the cells (Fig. 1B). Earlier reports also mentioned similar observations in PHB-producing bacteria (López-Cortés *et al.*, 2008; Sathiyarayanan *et al.*, 2013). Nile red stained cells showed numerous bright orange-red granules within the cells under fluorescence at an excitation wavelength of 561 nm indicating the accumulation of PHA (Fig.1C).

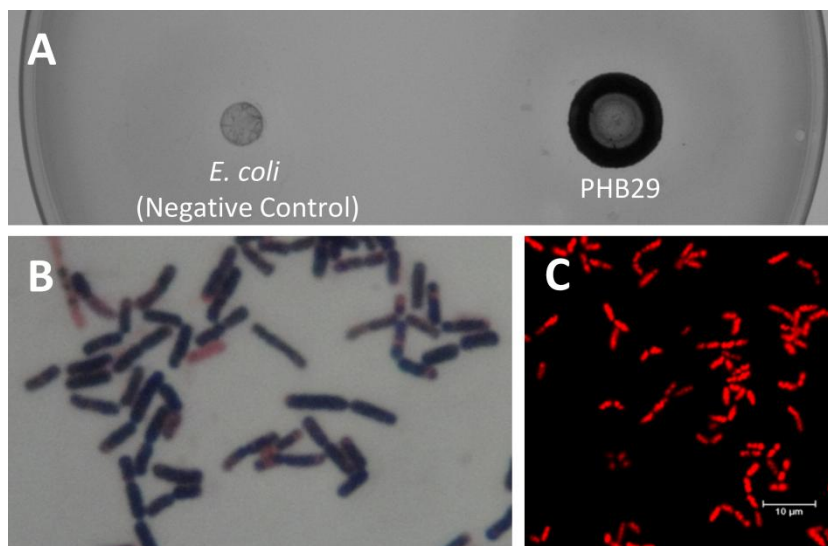
### **PHA production from starch**

When PHB29 was grown in starch containing medium, cell mass production and polymer accumulation were found to be increasing with increase in temperature, with a maximum cell dry mass (CDM) value of 3.07 g/L and a PHA yield of 73.46% (w/w) at 34°C and beyond this temperature, cell mass production as well as the polymer yield showed a decreasing trend (Fig. 2). From this, it is evident that this *B. megaterium* strain isolated from the forest soil has an optimum temperature for growth and polymer accumulation around 34 °C. This yield is much higher than earlier

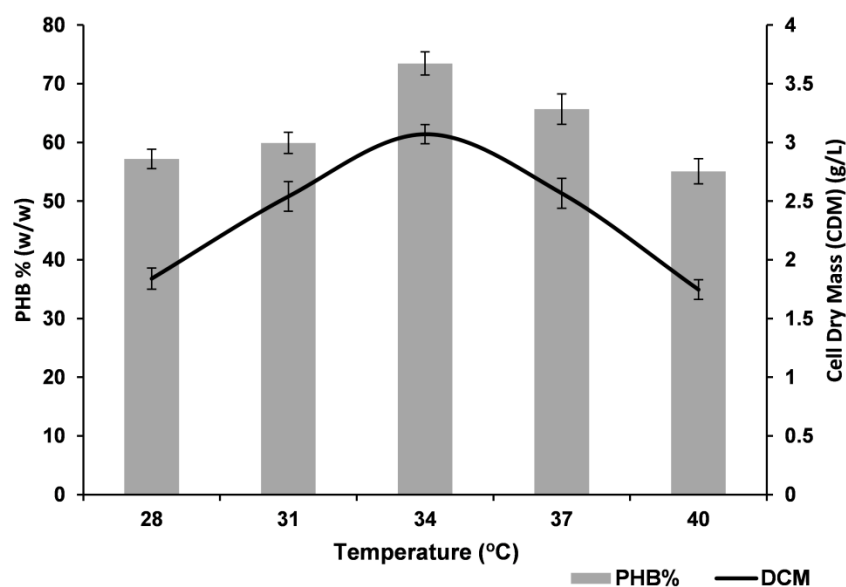
reports of PHB production from *B. megaterium* using sago starch and cassava starch (Krueger *et al.*, 2012; Yanti *et al.*, 2013). The polymer production attained a much better level than the earlier reports from other *Bacillus* spp., using starch feed stocks such as raw potato starch (34.68%

w/w), hydrolyzed cassava starch (29.7% w/w) and hydrolyzed jackfruit seed powder (29.32% w/w) (Krueger *et al.*, 2012; Ali and Jamil 2014; Gowda and Shivakumar 2014).

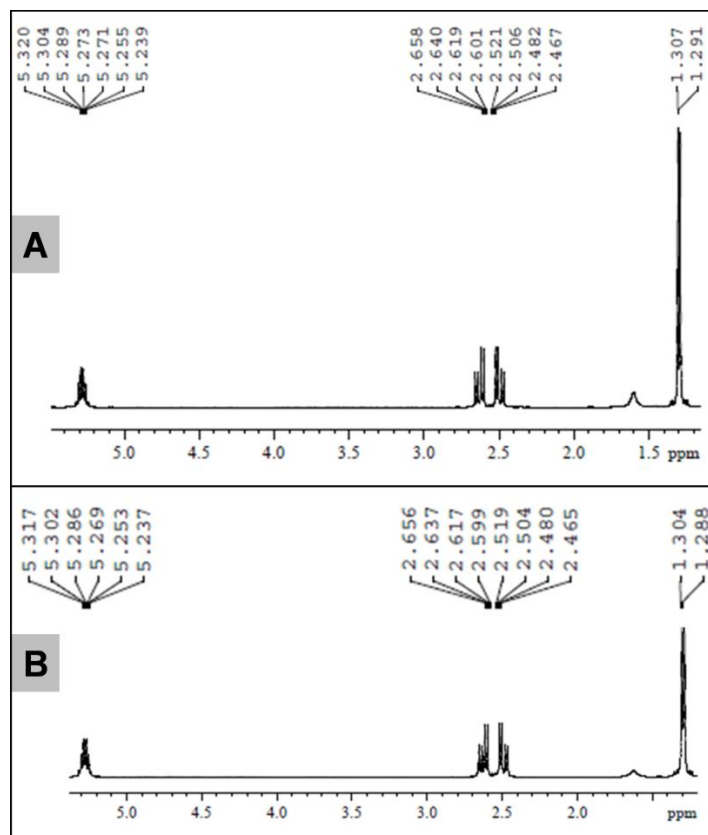
**Fig.1** A-Sudan Black B colony staining, B & C- Polymer accumulated PHB29 cells (B-Sudan Black, C- Nile Red)



**Fig.2** Growth and polymer production by PHB29 using starch as carbon feed stock at different temperatures



**Fig.3**  $^1\text{H}$  NMR spectra of PHB (A-polymer standard, B-polymer extracted from PHB29)



Utilising renewable and cheap carbon sources for PHA production is an ideal strategy for economical production of biodegradable plastics. Starch is a cheaper carbon source when compared to glucose and other refined carbon compounds. The higher efficiency of *B. megaterium* PHB29 to convert starch to PHB indicates that the bacterium possess a better amylolytic machinery working in tandem with PHB biosynthetic enzymes.

### Identification of the polymer

Figure 3 shows the  $^1\text{H}$  NMR spectrum of standard PHB (Sigma-Aldrich) as well as the polymer extracted from PHB29. It showed signals for a methine group (-CH-) between 5.23 and 5.32 ppm, a methylene group (-CH<sub>2</sub>-) between 2.46 and 2.65 ppm, and the methyl group (-CH<sub>3</sub>) between 1.28

and 1.30 ppm. These signals confirm the material as a homopolymer, Poly-3-hydroxybutyrate (PHB) (Salgaonkar *et al.*, 2013). It was previously reported that *B. megaterium* genome harbors Class IV PHA synthase gene which preferentially catalyzes the biosynthesis of short chain length PHAs such as PHB (McCool and Cannon, 2001).

In conclusion, soil bacteria are a rich source of highly efficient and novel enzymes with wide array of applications. *B. megaterium* PHB29 which was originally isolated from soil has efficient amylases along with PHB biosynthetic enzymes, which reflected in its superior starch to PHB conversion capacity than other reported *Bacillus* spp. Carbon sources contribute a major share in the production cost of PHAs and using inexpensive raw material such as starch



instead of glucose and other refined sugars is a good option for the cost effective production of PHAs. Starch based raw materials require a prior saccharification for effective bacterial fermentation. Employing PHA producers with good amyolytic activity can circumvent this pretreatment process of the substrate and thereby aid in the reduction of the production cost. Considering all these facts the bacterial strain described in the study is an ideal candidate for the cost effective, large scale production of PHB using starch as feed stock.

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