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Polyhydroxyalkanoates production by sugarcane rhizospheric soil bacterial isolate

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ABSTRACT

PHAs are the biopolymers synthesized by a wide array of bacteria as carbon and energy storage granules. However, its synthesis by sugarcane rhizospheric soil bacteria has gained the utmost attention due to its utilization of a broad spectrum of synthetic or inexpensive substrates. Herein, 20 aerobic bacteria were isolated from the rhizospheric soil of sugarcane using standard bacteriological techniques. Among them, 03 Gram positive bacterial isolates (B1, B2 & B3) were able to accumulate PHAs granule in their cytosol as confirmed from Sudan black B staining. Based on the intensity of staining, bacterial isolate B1 was selected for further study. Under solid-state fermentation (SSF), bacterial isolate B1 was found to produce 1.2 g/l of PHAs using sucrose as carbon source. The bacterial isolate B1 was identified as *Bacillus* sp. B1 by morpho-physiological characterization. Further, optimization of process parameters, characterization of PHAs and species level identification of the bacterial isolate is highly essential in this regard.

1. Introduction

Synthetic plastic production has become inevitable in the world and are used in different sectors of operations. It was estimated that about 187 million tonnes (Mt) of petroleum-based plastic are generated per year globally (Chavan *et al*., 2021). However, their excessive use because of their mechanical integrity, excellent durability, cost-effectiveness and easy production, it is accumulating in the environment leading to environmental pollution (Narayanan *et al*., 2020). Hence, these petroleum-based plastics need to be replaced by bioplastics. Polyhydroxyalkanoates (PHAs), act as a suitable alternative to conventional plastics produced by various microbes including bacteria, archaea, cyanobacteria and plants as energy storage granules (Maity *et al*., 2020). Depending on the presence of monomer, these are categorized into three categories viz., short chain length (*scl*), medium chain length (*mcl*) and long chain length (*lcl*) PHAs. The molecular weight of PHAs varies between 200,000 - 2000,000 Dalton depending on desired bacterial strain, fermentation conditions and substrate used in the bioprocess technology (Mohapatra *et al*., 2020). Among all, polyhydroxybutyrate (PHB), poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), poly (3-hydroxybutyrate-co-4-hydroxybutyrate) (P3HB4HB) and poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) have been produced on a mass scale for commercial applications (Maity *et al*., 2017; Mohapatra *et al*., 2015). PHAs possess characteristics similar to synthetic plastics whereas, these are biodegradable and biocompatible under natural conditions to produce CO_2 and H_2O. Therefore, it is produced industrially and used in a broad spectrum of end products, ranging from packaging to medical applications (Amaro *et al*., 2019).

Several research on bioplastics suggest that due to genetic stability, easy cultivation and fast-growing ability, bacteria are the key organisms for the production of PHAs (Mohapatra *et al*., 2017). All at once, they can be biodegraded

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by living organisms by the process of decomposition leading to the formation of very small compounds by microbial activity (Aragosa *et al*., 2020). Microbes inhabiting different ecological niches such as estuaries, marine water, rhizospheric regions, groundwater and sewage accumulate PHAs granules due to stress experienced by them. These locations are often rich in organic contents to support actively involved microorganisms for PHA accumulation to meet the metabolic energy requirement. *Bacillus licheniformis, Bacillus cereus, Bacillus badius* are some of the reported Gram-positive PHAs producing bacteria isolated from rhizospheric soil regions of different plants like rubber, sugarcane etc (Mohapatra *et al*., 2015). The sugarcane rhizospheric region is known to be a hot spot of microbial activities since roots release several different organic compounds inform of exudates and mucilage which serve as adequate nutrient supply for microbes (Mohapatra *et al*., 2015). Therefore, the rhizospheric region is a highly favorable habitat for the proliferation, activity and metabolism of numerous microbes. Moreover, the predominance of amino acids and growth factors required for bacteria, are readily provided by the root exudates in the rhizospheric soil region. On the other hand, *Bacillus* species are the predominant soil-inhabiting bacteria thatcan grow by utilizing cheap raw material for their growth and development as well as accumulating PHAs (Dash *et al*., 2014). In light of the above, an attempt has been made to study PHA production by sugarcane rhizospheric soil bacterial isolate.

2. Materials and methods

2.1. Isolation of rhizospheric soil bacteria

Rhizospheric soil samples of sugarcane were collected from the agriculture field, Dhauli, Bhubaneswar, Odisha. Representative samples were collected using a sterile vial and then transported aseptically to the laboratory for bacteriological analysis. The collected samples were processed in the laboratory at the earliest to isolate rhizospheric soil bacteria and to study their morphology and other characteristic features required for their generic level identification. Ten-fold dilution followed by a spread plate method was performed to isolate desired bacteria. The required amount of sterile nutrient agar (NA) medium was prepared and poured into ten different sterile petriplates. Then, 0.1 ml of serially diluted sample from each dilution was spread to the respective petriplates and incubated at 37^0C for 24 hours. The colonies of distinct morphological characters were individually picked up, sub-cultured on NA medium and incubated at 37°C for 24 hours to obtain the pure culture. The resulting pure cultures were preserved in NA slants at 4^0C and also maintained in glycerol stock at -20°C for further characterization.

2.2. Screening of PHAs producer

Screening of the PHAs producing bacterial isolates was conducted by the Sudan black B staining method under bright field microscopic imaging (Pati *et al*., 2020). However, before the screening, the bacterial isolates were induced to accumulate PHAs granules by growing in nitrogen-limiting mineral salt medium (MSM) and incubated at 37°C for 48 hr. Then, the bacterial smear was flooded by 0.3% (w/v in 70% ethanol) of Sudan black B staining for 15 minutes followed by Gram's decolorizer for a few seconds and then counterstained with safranin (5% w/v in de-ionized water) for 10 seconds. The slides were then washed gently, dried and observed under light microscope (1000X, Leica DM5000B).

2.3. Generic level identification of PHAs producer

The PHAs producing bacterial isolates were subjected to Gram staining followed by biochemical characterization using the VITEK 2 Compact system (BioMerieux, France) (Yasin and Mayaly *et al.,* 2020). In this system, generic-level identification of bacterial isolates was conducted based on 63 different biochemical tests.

2.4. PHAs production

PHAs production via solid-state fermentation (SSF) was carried out using plate culture method. Briefly, 1L of MSM agar medium with pH 9.0 was prepared in 50 different petriplates and inoculum (24 hours fresh culture containing 1.5 x 10^8 cells/ml) was added following the lawn culture method and incubated at 37ºC for 72 hours. Then, PHA was recovered by following sonication and di-solvent extraction method. The harvested bacterial cell biomass was collected in 30 ml of acetone and the suspension was sonicated at 20 KHz/ power 100/ pulse 30s for 15 minutes. The cell biomass and filtrate (acetone extract) were collected, suspended in chloroform and evaporated at 70-80°C in a water bath to obtain PHAs film. Then, PHAs production (%) was estimated using the following formula (Pati *et al*., 2020; Mohapatra *et al*., 2016).

$$\text{PHAs production (\%)} = \frac{\text{Weight of PHAs}}{\text{Cell biomass (DCW)}} \times 100$$

3. Result and discussion

The rhizospheric soil region of sugarcane contains varied micro-flora due to the secretion of root exudates having adequate amount of carbon sources and inadequate amount of other nutrients. A wide array of bacteria is known to accumulate PHAs in the microhabitat where carbon concentration is higher and nitrogen content is lower

(Mohapatra *et al.,* 2015). These bacteria have been reported from various environments, but only a few from the rhizospheric soil region of sugarcane. On account of that, 20 different bacteria were isolated from the rhizospheric soil region of sugarcane (*Saccharum officinarum*). Among them, 03 bacterial isolates were found to accumulate PHAs in their cytosol as confirmed by Sudan black B staining (Fig. 1). Based on the intensity of staining, bacterial isolate B1 was selected for further study. Gram variability reactions revealed that, bacterial isolate B1 is Gram positive and rod in shape. Further, the bacterial isolate B1 was identified as *Bacillus sp.* B1 by morpho-physiological characterization.

Under SSF, the potent rhizospheric soil bacterial isolate *Bacillus sp.* B1 was found to produce 1.2 g/l of PHAs using synthetic carbon source. Our result coincides with recent data of PHAs production by different strains of *Bacillus* (Joseph *et al.,* 2021; Damle *et al.,* 2016; Mohapatra *et al.,* 2015; Dash *et al.,* 2014) through submerged fermentation process (SmF). However, reports are not available in the public domain for PHAs production via SSF using rhizospheric soil bacterial isolates. Researchers are still on their best path to isolate, identify and characterize potent PHAs producing bacterial isolates for better convenience in near future as these biopolymers represent a potential alternative to petrochemical-based plastics.

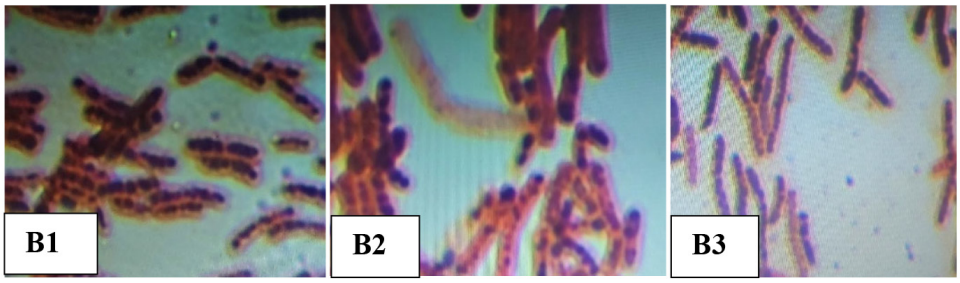

Fig 1: PHAs accumulating bacterial isolates B1,B2 and B3 under Sudan Black Bstaining

4. Conclusion

Under solid-state fermentation, the rhizospheric soil bacterial isolate *Bacillus sp.* B1 was found to produce 1.2 g/l of PHAs. However, optimization of process parameters, characterization of PHAs and species level identification of the bacterial isolate is highly essential in this regard.

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