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Screening and production of bioplastic (PHAs) from sugarcane rhizospheric bacteria

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Abstract

There has been considerable interest in the development and production of biodegradable polymer to solve the current problem of pollution caused by the continuous use of synthetic polymer of petroleum origin. Polyhydroxyalkanoates (PHAs) are known to be accumulated as intracellular inclusion in some bacteria. The materials properties exhibited by PHAs, ranging from stiff, brittle to rubber-like makes it a close substitute for the synthetic plastic. Under this study, ten bacteria were isolated from sugarcane rhizosphere sample collected from Pacheda, Raipur (Chhattisgarh). Isolates were grown in the production media containing high carbon concentration. Out of ten bacteria, only six bacteria produce PHA i.e. *Micrococcus luteus 2, Micrococcus mucilaginosus, Micrococcus nishinomiyaensis, Micrococcus radiodurans, Streptococcus equinus, Streptococcus raffinolactis.* Out of which, *Micrococcus mucilaginosus* and *Streptococcus raffinolactis* produce high amount of PHA. Because of their special characteristics and broad biotechnological applications, it can be industrially exploit for bioplastics production.

Keywords: Polyhydroxyalkanoates (PHAs), rhizosphere, soil, sugarcane

INTRODUCTION

Since synthetic plastics marked their debut in the 1950s, they have emerged to be among the most needed material in our daily life. These petroleum-based plastics are very stable in harsh conditions especially against the attack of chemical degradation and microbial decomposition, thus rendering them durable, highly resistant and 'blessed' with a very long life span in the environment. Due to their excellent properties and wide range of application, synthetic plastics have since championed the commodity market and the technologies related to plastics manufacturing are very well established. Because of the increasing environmental problems associated with discarded plastics many studies have been directed towards the development of a suitable eco-friendly material that can replace at least some of the commodity plastics. Polyhydroxyalkanoates (PHAs) are a class of biobased polymer with properties that closely resemble the properties of synthetic plastics currently dominating the market. Both PHAs and synthetic plastics are thermoplastics, moldable, and could be tailor-made for numerous applications ranging from stiff packaging goods to highly elastic materials for coatings.

In addition, the quality that sets PHAs apart from conventional plastics is the complete biodegradability of PHAs in the environment [13]. In the present investigation aimed to estimate the microbial community composition using PIB win software and quantification for PHA production so that with the help of these organisms we can exploit bioplastic production and made our environment eco-friendly.

METHODOLOGY

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Sample collection and preservation

Soil samples were collected from the agricultural field of sugarcane rhizosphere, of Pacheda, Raipur (C.G.) India. Soil sample was collected from 5-15 cm depth by gently scrapping of soil by using sterile needles in an airtight sterile polythene bags assigning them a collection number and site of collection. Sample was brought to the laboratory (Modern Biotech Research Lab, Raipur, Chhattisgarh, India-492010) and stored at 4°C till use.

Isolation of bacteria

Isolation of bacteria was performed by dilution plate technique [15] and direct plate technique, using nutrient agar medium (peptone 5g; beef extract 3g; NaCl 5g; agar 20g; distilled water 1000 ml; pH 7.2). One gram of dried soil sample was taken in 9 ml of sterile distilled water and mixed to a homogenous solution. Different dilutions of soil were prepared as 10^{-3}, 10^{-5} and 10^{-6} and were applied onto agar plates. The agar plates were prepared by addition of approx. 20 ml melted medium. After gently rotating, the plates were incubated at 28°C for 24 to 48 h. After incubation counted the colony-forming unit (CFU) with the help of Colony counter, Labtronics Inc, Canada. Selected colonies of bacteria were transferred from mother culture plates onto respective agar plates, incubated at 37°C for 24-48h. Pure cultures were stored at 4°C until further examination.

Bacterial colonies were characterized morphologically, biochemically and physiologically following the directions given by the Bergey's Manual of Systematic Bacteriology [11, 1]. Cultural characteristics of pure isolates in Nutrient agar media were recorded after incubation for 24-48h at 37°C. The patterns of growth to be considered were evaluated in the following manner: size, pigmentation, configuration, margin, elevation.

Identification of bacteria using probabilistic identification of bacteria (PIB) Win software

PIBWin (Probabilistic identification of bacteria) is one of the data base developed for the identification of bacteria based on numerical

taxonomy. In this data based equal weight is given to various cultural, morphological, physiological and biochemical features [3, 6].

Screening of polyhydroxyalkanoates (PHAs)

Minimal Salt Media (MSM) was prepared by (sodium chloride 3g; di-potassium hydrogen ortho phosphate 1.5g; Potassium di-hydrogen ortho phosphate 1.5g; magnesium sulphate 1g; sucrose 5g; ammonium nitrate 0.5g; distilled water 1000 ml; pH 7) for the production of polyhydroxyalkanoates. 50ml of MSM broth was taken in each ehrlemeyer flasks and autoclaved at 121°C for 20 min. 100µl of fresh bacterial inoculum were inoculated in each flasks and incubated for a week at 37°C.

Quantification of polyhydroxyalkanoates was performed by sodium hypochlorite method [2]. For extraction of PHAs, equal volume i.e. 1ml of bacterial suspension and 1ml sodium hypochlorite was taken in the culture-tubes, mixed well and were incubated for 1 hour at 37°C. After incubation, 1ml of acetone was added to the tube containing bacterial suspension and sodium hypochlorite, mixed well and was again incubated for 1 hour at 37°C for precipitation. Then, the tubes were centrifuged at 5000 rpm for 10-15 minutes at room temperature. After centrifugation, supernatant was discarded and the pellet was washed for 2-3 times by alcohol and distilled water. After washing the weight of pellet was measured. Weight of each filter paper was taken and then placed it on a Buchner funnel. Poured the contents of the flask into the funnel and the contents are allowed to completely drained off. Now the filter paper along with the pellet is transferred to an oven for drying. Reweighed the dried filter paper along with pellet. Weight was determined by substracting the initial weight of filter paper.

RESULTS

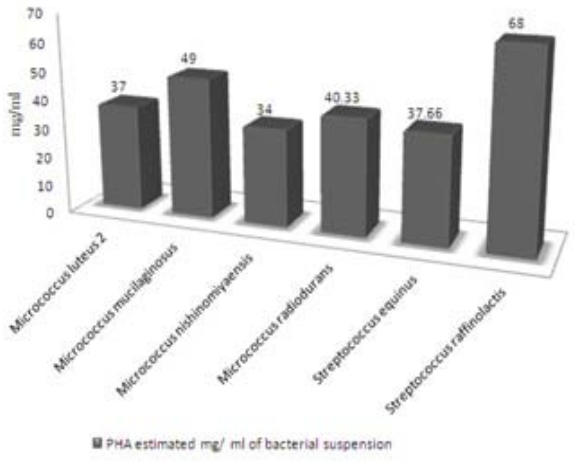
Summarized in Table: 1, Figure: 1.

Total of 10 bacteria were recorded from rhizospheric soil of Pacheda, Raipur (C.G.) India, using nutrient agar medium. Selected bacterial isolates were characterized by morphological, physiological and biochemical tests. PIB-win (Probabilistic identification of bacteria) identification system was used to identify the selected isolates i.e. *Arthrobacter species*, *Micrococcus halobius*, *Micrococcus luteus* 1, *Micrococcus luteus 2, Micrococcus luteus* 4, *Micrococcus mucilaginosus*, *Micrococcus nishinomiyaensis, Micrococcus radiodurans, Streptococcus equines*, *Streptococcus raffinolactis* (Table 1). All the isolates were grown in the production media and six bacteria produce polyhydroxyalkanoates i.e. *Micrococcus luteus* 2, *Micrococcus mucilaginosus, Micrococcus nishinomiyaensis, Micrococcus radiodurans, streptococcus equinus and Streptococcus raffinolactis.* PHA was extracted from these six bacteria (Fig. 1) in which *Micrococcus mucilaginosus* and *Streptococcus raffinolactis* produce high amount of PHA whereas other bacteria produce less amount of PHA.

S.No.	Culture code**	MB1	MB2	MB3	MB4	MB5	MB6	MB7	MB8	MB9	MB10
1.	Colony colour*	S	S	G	P	S	W	W	S	B	S
2.	Gram staining	+ cocci	+ cocci	+ cocci	+ cocci	+ cocci	+ cocci	+ cocci	+ cocci	+ cocci	+ cocci
3.	Indole production	–	–	–	–	–	–	–	–	–	–
4.	Methyl-red	–	–	+	+	–	+	–	+	+	–
5.	Voges-Proskaeur	–	–	+	–	+	–	+	+	+	–
6.	Citrate utilization	+	+	–	–	+	–	–	–	–	+
7.	Catalase	+	+	–	+	+	–	+	+	–	+
8.	H_2S production	–	–	–	–	–	–	–	–	–	–
9.	Urease	–	–	–	–		–	–	–	–	–
10.	Starch hydrolysis	–	–	–	–	–	–	–	–	–	–
11.	Oxidase	+	+	–	–	–	+	+	+	–	+
12.	O-nitrophenyl-ß-D-galactopyranoside	–	–	–	–	–	–	–	+	–	+
13.	D (+) Fructose	+	–	+	–	+	+	–	–	+	+
14.	D (+) Mannose	–	–	+	–	–	+	–	–	–	+
15.	Rhamnose	–	–	–	–	–	–	–	–	–	+
16.	Inositol	–	–	–	–	–	–	–	–	–	+
17.	Mannitol	–	–	–	–	–	–	–	–	–	+
18.	Sorbitol	–	–	–	–	–	–	–	–	–	+
19.	DNase	+	–	–	–	–	–	+	–	–	–
20.	Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–
21.	Gelatin liquefaction	+	+	–	+	–	+	–	+	–	–

*Colony colour - P=Pale white, W=White, G=Grey, S=Slimy, B=Blue.

**Name of isolates : MB1- *Micrococcus radiodurans*, MB2- *Micrococcus luteus 2*, MB3- *Streptococcus raffinolactis*, MB4- *Micrococcus nishinomiyaensis*, MB5- *Micrococcus luteus* , MB6- *Micrococcus mucilaginosus*, MB7- *Micrococcus luteus 4*, MB8- *Arthrobacter species*, MB9- *Streptococcus equinus*, MB10- *Micrococcus halobius*.



DISCUSSION

Soil is a heterogeneous, discontinuous, and structured environment with a high diversity of microhabitants in which conditions can change rapidly [10]. Thus, bacteria in soil have to cope up with fluctuating – in time and space – biotic and abiotic stresses. Bacteria can improve their establishment, proliferation, and survival in competitive niches such as soil and the rhizosphere is the accumulation and degradation of PHA [9,5]. In general, conditions of suboptimal growth are conducive to the production of PHAs [8]. In present study, 10 bacterias were isolated from sugarcane rhizosphere and were screened, after screening six bacterias were PHA producing. In nature, prokaryotic micro-organisms respond to sudden increases in essential nutrients in their usually hostile environment by storing important nutrients for survival during prolonged period of starvation [13]. Lemoigne for the first time reported accumulation of PHAs as cytoplasmic inclusionsin Gram-positive bacterium *Bacillus megatrium* [7]. PHB inclusion has been found in many micro-organisms, such as representatives of Gram-positive and Gram-negative bacteria and also archaebacteria, as insoluble inclusion in cytoplasm [12].

Supporting data for PHA production in telluric environments were provided by Wang and bakken (1998) [16], who screened 63 soil bacteria for PHA production. They concluded that strains capable of producing PHA were not necessarily superior to those that lack this ability. Instead, survival ability is strain-specific and depended upon the growth conditions prior to starvation. Most PHA producing bacteria were found to belong to pseudomonad, coryneform, and bacillus groups. In addition to *Pseudomonas and Bacillus*, Usman *et al* (2007) [14]reported the isolation of soil PHA-producing bacteria belonging to the genera *Citrobacter, Enterobacter, Klebsiella, Escherichia*, all of them enterobacteria. Among symbiotic bacteria and plant growth-promoting rhizobacteria, PHA production has been reported in members of the genera *Rhizobium, Azospirillum, Herbaspirillum* and *Azotobacter* [4]. In the present studies, one bacterial species *Streptococcus equinus*, have been already reported for PHA production but five bacterial species *Micrococcus luteus 2, Micrococcus mucilaginosus, Micrococcus nishinomiyaensis, Micrococcus radiodurans, Streptococcus raffinolactis,* have shown very good and moderate level of PHA production respectively. These five species have possibly been reported with PHA production for the first time. No reports are available about such ability however it needs to be verified further.

CONCLUSION

Microbial PHAs have emerged as a complementary material for petrochemical based plastics. The development of PHA into a branch of bulk chemical industry will address at least three issues: shortage of petroleum for plastic materials, reduction of CO_2 emissions, and environmental protection. In conclusion, study of these bacteria may indicate their special role in PHA production. Because of their special characteristics and broad biotechnological applications, bioplastics are compounds with an extremely promising future.

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