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Study on Polyhydroxyalkanoates Production using Rhizospheric Soil Bacterial Isolates of Sweet Potato

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Abstract

In this investigation, a total of twenty-five bacterial strains were isolated from rhizospheric soil region of sweet potato during pre-harvesting period. Presence of PHB granules was confirmed by Sudan Black B staining in four strains out of all isolates. The results obtained from Gram staining, biochemical characterization and enzymatic analysis; all four strains were Gram positive rod and belong to genus Bacillus with negative trend in caseinase activity. Highest PHA production (64.53%) was observed in Bacillus P3 under optimized condition such as temperature 37^{0}C and pH 7.Then the potential PHA producing isolates were tested for multiple resistances to antibiotics showing an advantageous feature for the bio-technological production of PHAs. Further characterization of PHA was carried out by FTIR. Thus the potential bacterial isolates can be exploited for production of biopolymer.

Keywords: Biopolymer, Caseinase, FTIR, Rhizospheric, PHA

1. Introduction

Now-a-days, non-biodegradable waste materials have been a growing concern of scientific researchers in response to the problems in public health and environmental issues[1]. Consumption and degradation of plastic waste produces toxins which have a deleterious impact on environment[2]. Several research and development on biodegradable plastics suggesting that bacteria have the ability to produce bioplastic in the form of polyhydroxyalkanoates (PHAs)[3]. PHAs are natural, renewable and biocompatible biopolymers which can be made into plastic materials with properties similar to petrochemical plastics. It is an energy storage inclusion synthesized intracellularly in presence of excess carbon with limiting concentration of nitrogen, phosphorus, sulphur or oxygen essential for growth[4] and can replace synthetic plastics in pharmaceutical & industrial applications. The goal of the current study is to find out a potential bacterial strain for PHA production under optimized condition. In our present study, an attempt has been made for screening, production and characterization of PHA produced by rhizospheric soil bacterial isolates of sweet potato.

2. Method

2.1 Sampling and Isolation of Bacteria

Soil samples were collected aseptically from rhizospheric soil region of sweet potato during pre-harvesting period in the field of CTCRI, Bhubaneswar, Odisha. Samples were transported to the laboratory for bacteriological analysis. Chemicals and reagents used in the research work were obtained from Hi-Media Laboratories Pvt. Ltd, Mumbai. Then, the aerobic, heterotrophic bacteria were isolated in nutrient agar by tenfold serial dilution followed by spread plate technique and incubated at 30°C for 48 hours. Colonies of different morphologies were selected and pure culture was obtained by streak plate method and preserved by glycerol stock at 4°C for future use.

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2.2 Screening of Bacteria for PHA Production

Existence of PHA granule was determined by Sudan Black B staining[5]. Prior to staining, bacterial isolates were inoculated to MSM medium with high carbon and low nitrogen ratio for induction of PHA granule. Sudan black B satin is lipophilic in nature and accumulated inside the cells indicated as PHA inclusion. The cultures were thinly smeared on glass slides and stained with 0.3% of Sudan black. After 10min, the slides were washed gently under steady flow of distilled water and treated with xylene for few seconds. In the next step of staining, slide was allowed to flood with 0.5% of Safranin for 30 sec and viewed under microscope with 100x. The accumulation of PHA granules could be identified from thebluish black colour deposition[6].

2.3 Identification and Characterization of Bacterial Isolates

Identification and characterization of screened bacterial isolates were carried out on the basis of their colony characteristics, Gram variability, standard biochemical tests, sugar utilization tests, enzymatic activities and antibiotic sensitivity test required by Bergey's manual of systematic Bacteriology[7] and PIB Win software[8]. Enzymatic activity such as amylase, cellulase, pectinase, gelatinase, caesinase, lipase, chitinase and DNAase of screened bacterial isolates were studied by spot inoculations using pseudo-selective media.

2.4 Estimation of Temperature, pH Tolerance and Antibiotic Sensitivity

The temperature tolerance test was conducted by allowing bacterial isolates to grow at various temperatures. Bacterial isolates were inoculated to 10ml NB in different test tube and incubated at different temperature such as 23^{0}C, 30°C, 37°C, 44^{0}C, 51^{0}C for 24 hours. Then the CFU/ml was counted and the optimum temperature for individual bacterial isolates was determined. Similarly, pH tolerance test was conducted by inoculating bacterial isolates in to10 ml NB in different test tube and incubated at 37°C for 24 hours at varied pH from 5-9 with help of 1N HCl, 1N NaOH and digital pH meter. Then the CFU/ml was counted and the optimum pH for individual bacterial isolates was determined. The selected bacterial isolates were screened for the antibiotic resistance by following[9] disc diffusion method. The isolates were exposed to most common antibiotics to observe the effect of these antibiotics on the physiology of the PHA producing bacterial isolate.

2.5 PHA Production by Bacterial Isolates

Growths of the bacterial isolates were carried out using submerged culture technique[10] for preparation of seed culture. Then seed culture was grown in 1000 ml of sterile minimal salt medium for 4days at 30°C in a shaker incubator with 125 rpm. The cultures were centrifuged at 6,000 rpm for 10 minutes and the pellet was transferred into pre-weighed petriplates by dissolving it in distilled water. The pellet dried at 80°C and the cell dry mass (CDM) was calculated. The dried pellet was mixed with sodium hypochlorite solution in 1:5 ratios for cell hydrolysis and incubated at 37°C for 30 minutes followed by centrifugation at 6500×g for 15 minutes. Cell pellets were washed twice with water and diethlyether, acetone& methanol in 1:1:1 ratio to remove sodium hypochlorite solution by following centrifugation. The washed pellet obtained was extracted with boiling chloroform in 1:1 ratio and allowed to stand for 30 minutes and centrifuged at 10,000 rpm at 4°C for 20 minutes. The chloroform layer was transferred to a clean tube and was concentrated by using a rotary evaporator at 50 ^{0}C. PHA content was determined by; % PHA production = (Weight of PHA/ Weight of biomass) × 100.

2.6 Characterization of PHA by FTIR Analysis

The extracted PHA was analyzed for identification of its functional groups through FTIR spectroscopy along with the PHB standard procured from Sigma. KBr was added to the PHA samples and compressed the mixture into translucent sample discs. Further study can follow FTIR spectrometer (Perkin-Elmer RX I) observation with spectral range of 4000–400 cmto identify the functional groups of the sample[11].

3. Discussion

3.1 Identification and Characterization of Bacterial Isolates

Twenty-five bacterial isolates were obtained from the rhizospheric soil region of sweet potato during pre-harvesting period signifying high sugar content of the soil. Out of which, four isolates showed a positive response to

Sudan black staining. Gram staining followed by microscopic observation result indicated that, these bacterial isolates are Gram positive and rod shaped. Then the PHA producing bacterial isolates were identified as Bacillus Sp. P1, *Bacillus* sp. P2, *Bacillus* sp. P3 and *Bacillus* sp. P4 on the basis of biochemical tests and other standard microbiological tests (Table 1). Similar findings were also observed[12,13] while isolated sixteen different *Bacillius* sp. form rhizospheric soil region of different plants and *Bacillus cereus* from rubber plants respectively. Moreover *Bacillus* species are the predominant soil inhabiting bacteria which can grow by utilizing cheap raw material. This pre-harvesting period results are also in agreement with a great quantity of microorganisms in sugar cane crops able to accumulate PHAs due to selective pressure caused by high carbon: nitrogen ratio[14,15]. Additionally, utilization of various tested sugar (Table 2) and negative trend in caseinase enzyme activity (Table 3) also supports bacteria for PHAs accumulation as granule in their cytosol.

3.2 Estimation of Temperature, pH Tolerance and Antibiotic Sensitivity

The results from (Table 4) indicate that, the selected bacterial isolates were able to grow optimally at 37^{0}C and moderate growth was observed at 23^{0}C, 30^{0}C, 44^{0}C & 51^{0}C respectively. The CFU counts for respective isolates were 8.2x10^5, 8.7x10^6, 9.4x10^6 and 4.5x10^6; that corresponds to findings of[16,4] they observed that PHA producing bacterial isolates grew better at pH 7. This might be a result of molecular adaptation of these bacterial isolates as well as increases the activity of enzyme for optimal growth.

Table 1. Biochemical test of bacterial isolates

Sl. No.	Biochemical test	P1	P2	P3	P4
1	Growth at 10% NaCl	+	+	-	+
2	Hippurate hydrolysis	-	-	-	-
3	Anaerobic growth	+	+	-	-
4	MR-VP test	-	-	-	-
5	Citrate reductase	-	+	-	-
6	Starch hydrolysis	+	+	+	+
7	Oxidase reductase	+	-	+	-
8	Casein hydrolysis	-	-	-	-
9	Uerase hydrolysis	-	-	-	-
10	Nitrate hydrolysis	-	+	-	+
11	Esculin hydrolysis	+	+	+	-
12	Growth at 50^{o}C	+	+	+	+

*Bacterial isolates: P1, P2, P3& P4 - : Negative, +: Positive

Table 2. Sugar utilization of bacterial isolates

Sugars	P1	P2	P3	P4
Arabinose	-	+	-	-
Maltose	-	-	+	+
Dextrose	+	+	+	+
Mellibiose	+	+	+	+
Rhamnose	-	+	-	-
Sucrose	-	+	+	-
Cellobiose	-	-	+	-
Fructose	-	+	+	-
Galactose	+	+	+	+
Lactose	-	+	+	-
Mannose	-	+	+	+
Raffinose	-	+	-	+
Salicin	+	+	-	-
Xylose	-	+	-	-
Glucose	+	+	+	+

Table 3. Enzymatic activity of bacterial isolates

Enzyme	P1	P2	P3	P4
Caseinase	-	-	-	-
Pectinase	+	+	-	-
Cellulase	+	+	+	+
DNAase	+	-	+	+
Gelatinase	-	-	+	+
Amylase	+	+	+	+
Lipase	+	+	+	+

Similarly, pH 7 is the optimum for luxuriant growth (Table 5) of selected isolates and the CFU counts were8.4x10^5, 8.9x10^6, 9.6x10^7 and 4.6x10^{7}respectively.Optimum growth of isolates is 7, which is similar to the pH of the rhizospheric soil region.

All four bacterial strains were subjected to test for multiple antibiotic resistances. Table 6 results depicted that, the bacterial isolates showed sensitive to all the tested antibiotics, however resistant to erythromycin. This is positive sign with regards to application of these bacterial isolates for PHA production.

3.3 PHA Production by Bacterial Isolates

The data from our study also concluded that all the bacterial isolates were able to produce substantial amounts of PHA. The synthesis of PHA is expressed as percentage yield of the cell dry weight (Table 7). The PHA extracted from isolatesP1, P2, & P4 were60.16, 54.32 & 58.21% and highest PHA production (64.53%) was observed in Bacillus Sp. P3. Similar results were also observed by many researchers[17,18] that, Bacillus species has significant potentiality for PHA production under optimized condition.

Table 4. Effect of temperature on growth of bacterial isolate

Sl. No.	Bacterial isolates	(CFU/ml) 23°C	(CFU/ml) 30°C	(CFU/ml) 37°C	(CFU/ml) 44°C	(CFU/ml) 51°C
1.	*Bacillus* sp. P1	5.2 X 10^4	3.5 X 10^6	8.2 X 10^5	7.6 X 10^4	5.3 X 10^3
2.	*Bacillus* sp.P2	4.1 X 10^5	4.3 X 10^5	8.7 X 10^6	8.0 X 10^5	9.2 X 10^3
3.	*Bacillus* sp. P3	3.7 X 10^4	1.08 X10^5	9.4 X 10^6	7.2 X 10^5	4.7 X 10^3
4.	*Bacillus* sp.P4	3.1 X 10^5	1.35 X 10^7	4.5 X 10^6	8.4 X 10^4	3.2 X 10^3

Table 5. Effect of pH on growth of bacterial isolates

Sl. No.	Bacterial isolates	(CFU/ml)pH 5	(CFU/ml)pH 6	(CFU/ml)pH 7	(CFU/ml)pH 8	(CFU/ml)pH 9
1.	*Bacillus* sp. P1	5.1 X 10^3	6.9 X 10^5	8.4 X 10^5	5.1 X 10^5	5.0 X 10^5
2.	*Bacillus* sp.P2	2.09 X 10^5	2.5 X 10^6	8.9 x 10^6	8.2 X 10^5	6.9 X 10^4
3.	*Bacillus* sp.P3	3.09 X 10^5	6.5 X 10^5	9.6 X 10^7	6.1 X 10^5	9.7 X 10^3
4.	*Bacillus* sp.P4	1.7 X 10^4	2.21 X 10^6	4.6 X 10^7	3.5 X 10^6	2.7 X10^5

Table 6. Antibiotic sensitivity test of bacterial isolate

Antibiotics	P1	P2	P3	P4
Polymixin B	+	+	+	+
Streptomycin	+	+	+	+
Chloramphenicol	-	+	+	+
Ampicilin	+	+	+	+
Bacteriocin	+	+	+	+
Gentamycin	+	+	+	+
Penicillin	-	+	+	+
Cephalidroxil	+	+	+	+
Cephadrin	+	+	+	+
Doxycyclin H	+	+	+	+
Erythromycin	-	-	-	-
Kanamycin	-	-	+	-
Amphotericin	+	+	-	-

3.4 Characterization of PHA by FTIR Analysis

PHA polymer produced using essential substrates present on as growth medium was investigated for identification of its functional groups (Figure 1) through FTIR spectroscopy. From the spectra, the extracted sample were observed at 1732 cm^{-1} which corresponds to(C=O stretch) and other vibrational picks at 2346 cm^{-1}(O-H stretch), 2175 cm^{-1}(C≡N), 1527 cm^{-1}(N-O) and 1070 cm^{-1}(C-N) indicates presence of PHA[19].

4. Conclusion

The isolate characterized in this study *Bacillus* sp. P3 possesses the potential for the production of PHB (64.53%) *in vitro*. It is pertinent to mention that *Bacillus* species are dominant bacteria in industry because they can grow in chief raw material, rapid growth rate leading to short fermentation cycle times and secretion of hydrolytic enzymes. Thus, the potential bacterial isolate will be subjected to molecular characterization and further investigated to increase the productivity of PHB by supplementation of chief raw material in order to reduce the cost of upstream & downstream processing.

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Table 7. PHA production by bacterial isolates

Sl. No.	Bacterial isolates	Biomass (gm.)	PHA (gm.)	PHA(%)
1	*Bacillus* sp.P1	1.225	0.737	60.16
2	*Bacillus* sp. P2	1.632	0.887	54.32
3	*Bacillus* sp.P3	1.469	0.948	64.53
4	*Bacillus* sp. P4	1.716	0.999	58.21

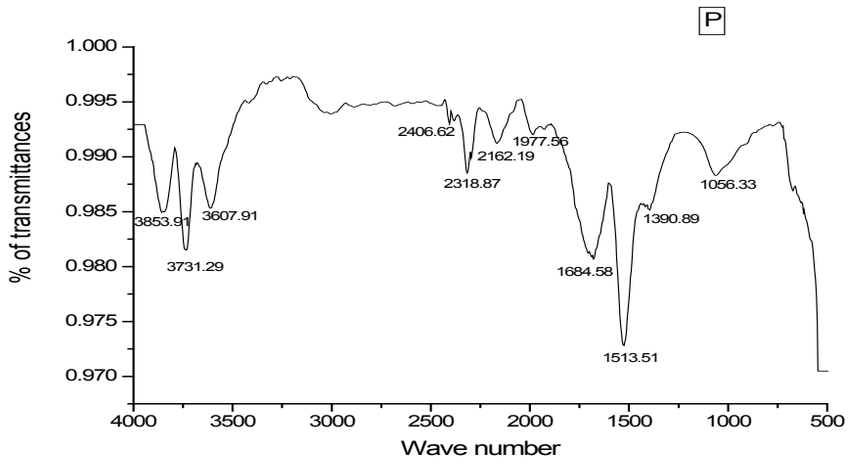

Figure 1. FTIR analysis of PHA sample extracted from *Bacillus* sp. P3.

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