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Production of Polyhydroxyalkanoates by Sugar Cane Rhizospheric Soil Bacterial Isolates

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In this research work, eight bacterial isolates were obtained from the rhizospheric soil region of sugarcane. Then the isolates were screened for the presence of PHA granule by following Sudan black staining of which, four bacterial isolates showed positive to Sudan black staining. However, the bacterial isolates showing PHA granule were Gram positive rod. Biochemical characterization revealed that all the bacterial isolates belongs to genus Bacillus. A negative trend in cellulase and pectinase activity were observed in case of all the PHA producing bacterial isolates. The optimum temperature and pH of all the bacterial isolates were found be 7 and 37^{0}C respectively. Under optimized condition *Bacillus licheniformis* produces 53.01% of polyhydroxyalkanoates in MSM culture medium. The production of PHA was found to be increased along with the increase in the biomass. The FTIR analysis of the extracted PHA showed the distinct peaks corresponding to C=O groups and the spectroscopic analysis gave proper insight for the chemical structure of PHB by reflecting the monomeric units. Further studies are required for elucidation of structure of the monpmer and to reduce the cost of production.

Key words: Rhizospheric, Sudan-black, Biochemical, Polyhydroxyalkanoates & MSM.

Polyhydroxyalkanoates (PHAs) are a complex class of biopolymers, synthesized by bacteria as storage compounds for carbon and energy. These are synthesized in the presence of excess carbon and with at least one nutrient essential for growth such as nitrogen, phosphorus, sulphur or oxygen present in limiting concentration[1]. PHAs exhibit a high degree of polymerization and molecular weights up to several million dalton[2]. It is biodegradable, insoluble in water, nontoxic, biocompatible, piezoelectric and thermoplastic. These features make them suitable for several applications in the packaging industry, medicine, pharmacy, agriculture, food industry[1,3]. PHAs can be obtained from renewable resources, thus a remarkable intrest has been increased for commercial production of polymers. However chemical synthesis of these polymers is difficult and not economically feasible. Hence, micro-organisms are the alternate source from which the polymers can be obtained at low cost and high purity. PHA accumulation is one of the responses towards stress experienced by microorganisms residing at different ecological niches such as estuarine sediments, marine habitat, rhizosphereric soil, groundwater sediments and sewage. These environments are often rich in organic contents and less rich in nutrient contents support the microbial population actively involved in PHA accumulation to meet the metabolic energy requirements during starvation period[4]. Though different types of PHAs are available however material properties and potential application of the PHAs vary depending on the monomer composition[5]. Many Gram negative and Gram positive bacteria are capablility to produce PHAs,

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however Gram positive bacteria lack of LPS lyer and are consider to be the most potential microbes for PHAs production[5]. The genus Bacillus is common with many other PHA-accumulating Gram-positive bacteria[6,7] accumulates co-polymers of 3HB when grown on different substrates. Moreover in this research work Bacillus species were isolated from rhizospheric soil region of sugar cane and studied for PHAs production.

MATERIALS AND METHOD

Sample collection

Representative soil samples were collected form rhizospheric region of sugar cane for selective isolation of PHA producing bacteria. The samples were collected in sterile plastic bottle and were transported to the laboratory aseptically for further analysis.

Isolation and preservation of bacterial isolates

The samples were processed in the laboratory for isolation of bacteria using standard procedures of serial dilution and spread plating. Colonies of distinguished morphologies were individually picked and sub-cultured and preserved at 4°C for further use.

Screening of PHA producing bacterial isolates

Sudan Black B staining was used for detecting the existence of PHA in cytoplasm of bacterial cells by[8]. However, before screening, the isolates were induced to accumulate PHA in a nitrogen-limiting medium (MSM) for 24 hours. Smear was made on a clean glass slide, after drying, 0.3% of Sudan black was added. Then slide was washed gently with distilled water after 10 minutes. The dried slides were engrossed in xylene for few seconds and allowed to flood with 0.5% of Safranin for 30 sec, the slide were washed gently and observed under light microscope.

Identification and characterization of bacterial isolates

The morphological and physiological properties of the isolates were investigated on the basis of their colony characteristics on the Minimal salt agar that contained NaCl (3.0 g L^{-1}), KH_2PO_4 (1.5 g L^{-1}), K_2HPO_4 (1.5 g L^{-1}), $MgSO_4.5H_2O$ (1.0 g L^{-1}), glucose (10.0 g L^{-1}), ammonium nitrate (0.5 g L^{-1}), agar agar (5.0 g L^{-1}) and Gram's reaction. After the microscopic examination the Gram positive rods were processed for identification by the standard prescribed biochemical tests, enzymatic, sugar utilization and antibiotic sensitivity test required by Bergy's manual of determinative bacteriology[9] and PIBWin software[10].

Enzymatic activities of the bacterial isolates

All the PHA producing bacterial isolates were screened on pseudo-selective media for production of various industrially important extracellular enzymes like amylase, cellulase, pectinase, gelatinase, caesinase, lipase and chitinase, DNAase following standard microbiological methods.

Estimation pH tolerance

The pH tolerance test was conducted to find out the optimum pH for growth of bacterial isolates. Selected bacterial cultures were revived in nutrient broth. Ten ml of nutrient broth was taken in different tubes and the pH was adjusted from 5-9 with help of 1N HCl, 1N NaOH and digital pH meter. 100µl of the overnight culture was dispensed into the test tubes and incubated at 37°C for 24 hours. Then the CFU/ml was counted and the optimum pH was determined.

Estimation temperature tolerance

Bacterial isolates were selected basing on their presence of PHA granule in the cytoplasm in order to find out the optimum temperature for their growth. Selected bacterial cultures were revived in nutrient broth. Ten ml of nutrient broth was taken in different tubes and the temperature was varied from 23°C to 51°C. 100µl of the overnight culture was dispensed into the test tubes and incubated at different temperature (23°C, 30°C, 37°C, 44°C, 51°C) for 24 hours. Then the CFU/ml was counted and the optimum temperature was determined.

PHA production by bacterial isolates

PHA production and extraction by the selected bacterial isolates was observed by following sodium hypochlorite[11]. The selected bacterial isolates were grown in conical flask containing minimal salt medium on a shaker at 37ºC for 4days with an agitation rate of 125 rpm. After 4days, the bacterial culture was centrifuged at 6500×g, then the supernatant was discarded and the pellet was transferred into pre-weighed petriplates by dissolving it in distilled water. The plates were dried at 80ºC in hot air oven. The dried weight of the pellet was taken in order to know the weight of biomass. Then sodium hypochlorite

solution was added to the dried pellet in 1:5 ratios to remove non PHA materials, transferred to the centrifuge tubes and kept in shaker for 30 minutes at 37ºC. After incubation, the samples were centrifuged at 6500×g for 15 minutes. The supernatant was discarded, the pellet was washed with the distilled water and wash with diethlyether and acetone in 1:1 ratio to remove sodium hypochlorite solution by centrifugation. Then the pellet was collected, 10 ml of chloroform was added to the pellet and filtered into the pre-weighed petriplates. The chloroform gets evaporated which leaves the PHA film in the petriplates. The weight of the PHA film was observed and the PHA production was calculated by following formula, % of PHA production = (Weight of PHA/ Weight of biomass) × 100.

Characterization of PHA by FTIR analysis

The functional group present in the extracted PHA was determined by FTIR spectroscopy along with the standard PHB procured from Sigma. PHA sample was mixed with 2 % KBr. The mixtures were compressed into translucent sample discs and fixed in the FTIR spectrometer (Perkin-Elmer RX I) under the following conditions: spectral range, 4000–400 cm) 1; window material, CsI; 16 scans; resolution 4 cm) 1; the detector was a temperature-stabilized, coated FR-DTGS detector for analysis[12] .

RESULTS AND DISCUSSION

Identification and characterization of bacterial isolates

A total of eight bacterial isolates were obtained from the rhizospheric soil region of sugarcane. Out of all, four bacterial isolates showed positive to Sudan black staining. Then the bacterial isolates showing PHA granule were subjected to Gram's reaction and found that, all the bacterial isolates were Gram positive rod. Then the PHA producing bacterial isolates were identified on the basis of biochemical tests and other standard microbiological tests (Table 1). The identified Gram positive PHA producing bacterial isolates were *Bacillus licheniformis* ID score (0.980), *Bacillus cereus, Bacillus licheniformis* ID score (0.999) and *Bacillus badius*. Similar findings were also observed[13,14] while isolated sixteen different species of *Bacillius* form rhizospheric soil region of different plants and *Bacillus cereus* from rubber plants respectively. Moreover *Bacillius* species are the predominat soil ihabitig bacteria which can grow by utilising cheap raw material for their growth and development. This results are also in agreement with the literature regarding finding a

Table 1: Biochemical characterization of bacterial isolates

Sl. No.	Biochemical test	B1	B4	B6	B8
1	Growth at 10% NaCl	-	-	+	-
2	Hippurate hydrolysis	-	-	-	-
3	Anaerobic growth	-	+	+	+
4	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 50ºC	+	+	+	+

Table 2. Enzymatic activity of bacterial isolates

Enzyme	*B. licheniformis* ID score(0.980)	*B. cereus*	*B. licheniformis* ID score(0.999)	*B. badius*
Amylase	-	+	+	-
Caseinase	+	+	+	-
Chitinase	-	-	-	-
Gelatinase	+	+	+	+
Lipase	+	-	-	+
Cellulase	-	-	-	-
Pectinase	-	-	-	-
DNAse	+	-	-	+

- : Negative, +: Positiv

great quantity of microorganisms in sugar cane crops able to accumulate PHAs due to selective pressure caused by high carbon : nitrogen ratio[15,16].

Enzymatic activities of the bacterial isolates

A negative trend in sugar utilization such as cellulose, pectin and chitin was observed in case of all the bacterial isolate, however the bacterial isolates showed positive to gelatin (Table 2). Three of the bacterial isolates showed positive result for caesinase activity except *Bacillus badius*. *Bacillus cereus* and *Bacillus licheniformis* showed positive to amylase while negative result against DNase and lipase but *Bacillus licheniformis* and *Bacillus badius* showed positive for lipase activity while negative for amylase activity. This findings can be corroborate with the findings of [17] who observed hydrolysis of starch, cellulose, casein, lipid, gelatine, pectin and chitin for characterization of bacterial isolates of soil sample. The hydrolysis of various sugars by the bacterial isolates may be due to availability of different sugar in the rhizospheric region of sugar cane plant. Moreover it is a positive sign for PHA production by the selected bscterisl isolates using various sugars as source of carbon and energy.

Effect of pH on growth of bacterial isolates

The result of pH tolerance (Table 3). of the bacterial isolates such as *Bacillus licheniformis, Bacillus cereus, Bacillus licheniformis* & *Bacillus badius* suggested that,

Table 3. 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.	*B. licheniformis* IDscore (.980)	4.1 X 10^3	6.0 X 10^5	8.4 X 10^5	5.1 X 10^5	5.0 X 10^5
2.	*B. cereus*	1.99 X 10^5	2.5 X 10^5	8.9 x 10^6	8.2 X 10^5	6.9 X 10^5
3.	*B. licheniformis* IDscore(0.999)	1.05 X 10^5	6.5 X 10^5	9.6 X 10^7	7.5 X 10^4	9.7 X 10^3
4.	*B. badius*	1.57 X 10^4	2.21 X 10^4	4.6 X 10^7	2.95 X 10^5	2.7 X10^5

Table 4. Effect of temperature on growth of bacterial isolate

Sl. No.	Bacterial isolates	(CFU/ml) 23^{0}C	(CFU/ml) 30°C	(CFU/ml) 37°C	(CFU/ml) 44^{0}C	(CFU/ml) 51^{0}C
1.	*B. licheniformis* IDscore(0.980)	4.2 X 10^4	3.5 X 10^6	8.2 X 10^5	7.6 X 10^4	5.3 X 10^3
2.	*B. cereus*	5.8 X 10^5	4.1 X 10^7	8.7 X 10^6	8.0 X 10^5	7.2 X 10^3
3.	*B. licheniformis* IDscore(0.999)	3.5 X 10^4	1.08 X 10^7	9.4 X 10^6	7.2 X 10^5	4.7 X 10^3
4.	*B. badius*	3.8 X 10^5	1.65 X 10^7	4.4 X 10^6	8.2 X 10^4	3.2 X 10^3

Table 5. PHA production by bacterial isolates

Sl.No.	Bacterial isolates	Biomass (gm.)	PHA (gm.)	% of PHA
1	*B. licheniformis* IDscore (0.980)	0.825	0.437	53.01%
2	*B. cereus*	0.532	0.2487	46.76%
3	*B. licheniformis* IDscore (0.999)	0.669	0.2148	32.11%
4	*B. badius*	0.706	0.292	41.48%

Table 6. Peaks obtained by FTIR and their corresponding annotations

Peaks (cm-1)	3618	2331	1675	1714	1532
Bonds	O-H stretch, H-bonded	O-H stretch	–C=C– stretch	C=O stretch	N–Oasymmetric stretch
Corresponding functional groups	Alcohols, Phenols	Carboxylic Acids	alkenes	Alpha, betaunsaturated esters	Nitro compounds

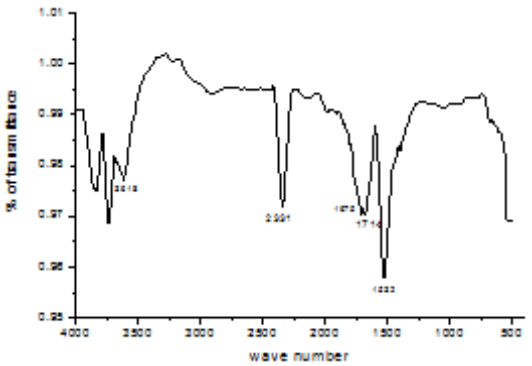

Fig. 1. FTIR analysis of the PHA sample extracted from *B. licheniformis* IDscore (0.980) showingcharacteristic peak for PHA.

the optimum pH for growth of the bacterial isolates were 7 and the CFU count were $8.4x10^5$, $8.9x10^6$, $9.6x10^7$ and $4.6x10^7$ respectively. However moderate growth was observed in pH 6 and 8. Again mild growth was also observed in pH 5 and 9. This result corresponds to that of[17,13] 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.

Effect of temperature on growth of bacterial isolate

Temperature tolerance of the bacterial isolates revealed (Table 4) that, optimum temperature for growth of all the bacterial isolates were 30^0C and the CFU count were $3.5x10^6$, $4.1x10^7$, $1.08x10^7$ and $1.65x10^7$ respectively. However moderate growth was observed at 37^0C and mild growth was observed at 23^0C, 44^0C & 51^0C respectively. This result corresponds to that of[17,13,1] they observed that PHA producing bacterial isolates grew better in between 25^0C to 30^0C. This might be a result of adaptation of these bacterial isolates to the natural habitat which was ranges between 25^0C to 35^0C.

PHA production by bacterial isolates

Under optimized condition the selected bacterial isolates such *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus badius* and *Bacillus licheniformis* were produced 0.825gm, 0.532gm, 0.669gm and 0.706gm of cell biomass respectively. The amount of PHA extracted from these bacterial biomass were 0.437gm, 0.2487gm, 0.2148gm, and 0.292gm per liter of MSM. However *Bacillus licheniformis* (ID score 0.998) is the highest PHA (53.01%) producting bacterial isolates (Table 5). The obtained result falls within the results obtained by[18,19,20]. In addition to that,[21] reported that, the PHA production in *Bacillus* sp. was found to be optimum with 0.5 g/l of nitrogen source, 120 rpm of agitation and 6.4 g/l of biomass inocolum which in accordance to the result obtained during the present study.

Characterization of PHA by FTIR analysis

The functional groups of the extracted PHA samples from the potent isolate *Bacillus licheniformis* (ID score 0.998) showed (Table 6) the characteristic peaks at 3618 cm-1 (H-bonded O-H stretch), 2331 cm-1 (O-H stretch), 1675 cm-1 (–C=C– stretch), 1714 cm-1 (C=O stretch), 1532 cm-1 (N–Oasymmetric stretch), High intense peaks were obtained at 3618 cm-1, 2331 cm-1, 1714 and 1675 cm-1, (Fig.1) however less intense peaks found at 3839 cm-1, 3735 cm-1. The spectroscopic analysis gave proper insight for the chemical structure of PHB by reflecting the monomeric units, which is predominantly present in the PHA polymer. The result obtained in this study is well within the result obtained by the previous workers [22,23,24,25]. The IR spectrum indicates the presence of monomeric units with a strong absorption band at 1714 cm^{-1} corresponding to C=O valence vibration of the thio-ester bond.

CONCLUSION

The isolate characterized in this study *B. licheniformis* IDscore (0.980) possesses the potential for the production of PHB (53.01%) in vitro. It is pertinent to mention that Bacillus species are dominant bacteria in industry for a variety of reasons including grow in chief raw material, rapid growth rate leading to short fermentation cycle times, secretion of hydrolytic enzymes and production of co-polymers from structurally unrelated sources. Thus, the potential bacteria *B. licheniformis* IDscore (0.980) is further investigated to increase the productivity of PHB by supplementation of chief raw material and reduction in the cost of upstream & downstream processing making the whole process more cost-effective.

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