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Evaluation of poly(3-hydroxybutyrate) production by microbiota endogenous to oleaginous plants

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ABSTRACT

Aims: Microbiota endogenous to oleaginous plants have attracted special attention in recent years for their biotechnological potentials and applications including the production of biodegradable biopolyester poly(3-hydroxybutyrate) [P(3HB)] as an alternative to thermoplastics. The present study is aimed to screen the endophytic bacteria of selected oleaginous plants such as *Arachis hypogaea* L., *Brassica napus* L., *Brassica nigra* L., *Helianthus annuus* L., *Ricinus communis* L. and *Sesamum indicum* L. for the production of P(3HB).
Methodology and results: Bacteria endogenous to the oleaginous plants were isolated from surface sterilized healthy tissues following sterilization with 70% ethanol and 0.5% sodium hypochlorite and screened for P(3HB) production in mineral salts medium. Nile blue A staining method was used for detection of intracellular P(3HB), while the accumulated biopolyester was quantified spectrophotometrically following chemical conversion to chrotonic acid by treating with sulfuric acid. Five potent P(3HB) accumulating isolates have been selected and identified as *Cellulosimicrobium cellulans* AHS 01 (KX458038), *Beijerinckia fluminensis* AHR 02 (KX458039), *Exiguobacterium acetylicum* BNL 103 (KX458037), *Bacillus toyonensis* BNS 102 (KX458036) and *Bacillus cereus* RCL 02 (KX458035) based on morphological, physio-biochemical and 16S rDNA sequence analysis. These endogenously growing bacterial isolates accumulated intracellular biopolyester accounting 43-62% of their cell dry weight (CDW) when grown in mineral salts medium supplemented with yeast extract. Intracellular accumulation of P(3HB) by these isolates have also been confirmed by FTIR spectral analysis of lyophilized cell mass and ^{1}HNMR spectra of the extracted polymer.
Conclusion, significance and impact of study: These findings, first of its kind point to exploration of endogenous bacterial communities of oil-producing plants as a potential bioresource for production of P(3HB) bioplastics in a sustainable manner.

Keywords: Endogenous microbiota, polyhydroxyalkanoates, poly(3-hydroxybutyrate), biodegradable polyester, oleaginous plants

INTRODUCTION

Bacteria endogenous to plants represent a class of endosymbionts that colonize intercellular as well as intracellular spaces of plant tissues without causing any morphological changes or disease symptoms. Endogenous bacterial association has been recorded in majority of the plant species so far studied and a wide diversity of bacterial taxa have been isolated and identified from amongst several agriculturally and medicinally important plant species (Strobel *et al.*, 2004; Miliute *et al.*, 2015). Such endophytic relationships are probably in existence from the time of appearance of plants on earth and might have led to exchange of genetic information between the endogenous bacterial population and the host plant. Endogenous bacterial communities have shown beneficial effects on their hosts by promoting plant growth, modulating plant metabolism and phytohormone signalling leading towards better adaptation to biotic and abiotic stresses (Mei and Flinn, 2010). Moreover, they play significant role in the defence of host plant against phytopathogenic attack by producing an array of novel secondary metabolites and bioactive compounds (Brader *et al.*, 2014). Significant research has been conducted in recent years to identify the endogenous microbiota from agricultural crop plants, and also to recognize their important metabolites for exploitation in medicine and industry (Sessitsch *et al.*, 2012; Miliute *et al.*, 2015).

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Diversity of bacteria harbouring the internal tissues of oleaginous plants such as *Brassica napus* L. (Graner *et al.*, 2003), *Olea europaea* L. subsp. *europaea* var. *sylvestris* (Aranda *et al.*, 2011), *Helianthus annuus* L. (Forchetti *et al.*, 2007) as well as *Arachis hypogaea* L. (Ibanez *et al.*, 2009) has been explored. Furthermore, endogenous bacterial species isolated from *Helianthus annuus* L. (Forchetti *et al.*, 2007) have been utilized for plant growth promotion. Likewise, lipid-rich tissues of oleaginous plants which provide unique ecological niche and microenvironment for growth and sustenance of endogenous bacteria may possibly synthesize and accumulate lipoidal substances. Zhang *et al.* (2014) have reported the presence of an oleaginous bacterium endophytic to thin shelled walnut, which synthesized and accumulated 40% (w/v) lipid in culture utilizing cotton-stalk hydrolysate. Similarly, endophytic fungi isolated from oleaginous crop have shown high lipid accumulation with potential for biodiesel production (Peng and Chen, 2007).

Poly(3-hydoxybutyrate) [P(3HB)], the simplest and most common polyhydroxyalkanoates (PHAs) is synthesized and accumulated as inclusion bodies in many bacteria when grown under unbalanced conditions. This unique polymer has been identified as environmentally biodegradable plastic of commercial value due to their physical and material properties analogous to synthetic plastics (Steinbüchel *et al.*, 1998). Wild type bacteria isolated from soil, activated sludge, marine ecosystems as well as those associated with plants have been found to be efficient P(3HB) producers (Khardenavis *et al.*, 2005; Gasser *et al.*, 2009). Moreover, development of newer fermentation strategies along with use of non-conventional renewable carbon sources including different plant oils has significantly reduced the cost of PHAs production making the process economically viable (Lee *et al.*, 2008; Marsudi *et al.*, 2008; Gholami *et al.*, 2016). On the contrary, no systematic studies have so far been conducted to explore the possibility of utilizing the bacteria endogenous to oil-yielding crops for the synthesis and accumulation of polyhydroxyalkanoates.

In India, the oleaginous plants viz. *Arachis hypogaea* L., *Brassica napus* L., *B. nigra* L., *Helianthus annuus* L., *Ricinus communis* L. and *Sesamum indicum* L. are mass cultivated for commercial production of oils of different types. The economical significance and little or no systematic phytosphere microbiological studies of these plants justify the selection of these oleaginous plants for the present study. Here, in this communication, we report for the first time a systematic survey of the diversity of bacteria endogenous to these oleaginous plants and screening them for the *in vitro* synthesis and accumulation of P(3HB) under batch cultivation.

MATERIALS AND METHODS

Isolation and maintenance of microbiota endogenous to oleaginous plants

The selected oleaginous plants, *Arachis hypogaea* L. (Groundnut), *Brassica napus* L. (Canola), *Brassica nigra* L. (Black mustard), *Helianthus annuus* L. (Sunflower), *Ricinus communis* L. (Castor) and *Sesamum indicum* L. (Sesame) collected from Hooghly, Howrah and North 24-Parganas districts of West Bengal, India were used for the isolation of endogenous microbiota.

Fresh and healthy leaf, stem and roots of the collected oleaginous plants were washed thoroughly under running tap water and surface sterilized by immersing in 70% ethanol (2-3 min), 0.5% sodium hypochlorite (3-5 min) and again in 70% ethanol for 30 sec following the method of Reinhold-Hurek and Hurek (2011). This was followed by repeated washing of plant segments in sterile distilled water for at least three times. Samples were blot dried, cut aseptically into thin sections and plated on R2A agar, glycerol asparagine agar, nutrient agar and tryptic soy agar plates. The plates were incubated at 28-30 °C for 2-4 days and observed for growth of bacterial colonies surrounding plant tissue sections. Phenotypically distinguishable bacterial isolates were obtained in pure form and maintained at 4 °C on slopes of tryptic soy agar.

Diversity of culturable microbiota endogenous to oleaginous plants

Colonization frequency of bacteria endogenous to oleaginous plants was calculated as the total number of plant segments yielding bacterial colonies divided by the total number of segments incubated. Isolation rate was determined as the number of bacterial isolates obtained from the plated plant segments divided by the total number of segments incubated. The Shannon Weaver diversity index (H) was calculated as: $H = -\sum Pi \times \ln Pi$, where, Pi is the proportion of individuals that species "i" contributes to the total. Simpson's diversity index H_S was calculated as: $H_S = 1-[\sum n(n-1)]/N(N-1)]$, where, n is the total number of organisms of a particular species and N is the total number of organisms of all species.

Screening of bacterial isolates for P(3HB) production

Growth associated production of P(3HB) by the bacterial isolates was evaluated in glucose (2%, w/v) containing mineral salts medium (Ramsay *et al.*, 1990). The medium (50 mL/250 mL Erlenmeyer flask) was inoculated with freshly prepared inoculum at 2% level and incubated at 32 °C on a rotary shaker at 120 rpm. Growth and production of P(3HB) by the isolates were determined by qualitative and quantitative methods.

For detection of intracellular P(3HB) accumulation, cells were harvested (centrifugation at 10000×*g* for 10 min) after definite period of incubation, washed thoroughly with distilled water, smeared on a clean grease-free slide and stained with aqueous solution of Nile blue A at 55 °C for 10 min. The excess stain was removed by washing with distilled water, 8% (v/v) acetic acid and rinsed with water (Ostle and Holt, 1982). After blot drying the smear was examined under fluorescence microscope (Leica DM IL LED) with an I_2 filter.

Growth of these isolates was estimated by measuring the cell dry weight, while, the P(3HB) content of the

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acetone dried cell mass was quantified following extraction in warm (45-50 °C) chloroform (Ramsay *et al.*, 1990). The chloroform extract was evaporated to dryness, treated with concentrated H_2SO_4 in a boiling water bath for 10 min (Law and Slepeckey, 1961), cooled to room temperature and the absorbance was recorded at 235 nm using a UV-VIS spectrophotometer (Jenway, Model 6505). The amount of P(3HB) was quantified from the calibration curve prepared in the same way using authentic P(3HB) from Sigma, USA and expressed as percentage of cell dry weight.

Characterization and identification of bacterial isolates

The pure cultures of selected endogenous bacterial isolates were characterized and tentatively identified following standard morphological and physio-biochemical tests (Gerhardt *et al.*, 1994) including susceptibility to different antibiotics. Morphology of the cells were determined by phase contrast (Carl Zeiss No. 288997) and scanning electron (Zeiss Evo18 SEM) microscopy.

The 16S rDNA sequence of the selected bacterial isolates was determined by direct sequencing of PCR amplified 16S rDNA. The genomic DNA was isolated and purified according to the modified method of Marmur (1961) and the 16S rDNA was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR amplified product was purified using QIAquick gel extraction kit (Qiagen, Netherlands) and the sequencing reaction was performed with ABI PRISM Dye Terminator cycle-sequencing ready reaction kit (Applied Biosystems). The sequencing products were purified and electrophoresed on polyacrylamide sequencing gel using an ABI 377 automated DNA sequencer. Sequencing data were analyzed by ABI version 3.0.1 b3 software and compared with reference sequences using the NCBI BLASTN programme. Multiple sequence alignments were carried out by using BLOSUM 62 matrix with the program package Clustal-W employing the neighbor-joining algorithm method (Saitou and Nei, 1987) with MEGA version 6.0.

FTIR spectral analysis

For Fourier transform infrared (FTIR) spectroscopy, cells were harvested from the growing culture by centrifugation (10,000×*g* for 10 min), washed thrice in distilled water and lyophilized at –56 °C in a Secfroid lyophilizer (LSL). The lyophilized cell mass and the authentic P(3HB) samples as KBr pellets were scanned in a Perkin Elmer RX-1 FTIR spectrophotometer in the range of 4,000 to 500 cm^{-1} (Kamnev *et al.*, 2002).

^{1}HNMR spectral analysis

The polyester samples isolated from the cell mass were purified by precipitation with chilled diethyl ether. The polyesters recovered by centrifugation were dissolved in deuterochloroform ($CDCl_3$) and subjected to proton nuclear magnetic resonance (^{1}H NMR) spectroscopic analysis in a Bruker AV300 Supercon NMR spectrophotometer working in digital mode. A multinucleate probe head at 30-degree flip angle was used. The chemical shift-scale was in parts per million and tetramethylsilane (Me_4Si) was used as the internal standard (Doi *et al.*, 1989).

RESULTS

Isolation of culturable endogenous microbiota

Surface sterilized leaf, stem and root segments of the oleaginous plants incubated on R2A agar, glycerol asparagine agar, nutrient agar and tryptic soy agar plates showed growth of morphologically distinguishable bacterial colonies adhered to the surface of sterilized segments after 2-4 days of incubation. Avoiding the repetitive strains, a total of 154 phenotypically distinguishable bacterial isolates were obtained in pure form, the highest number (45) being derived from *B*. *nigra* L. (Table 1). The leaves of *B*. *nigra* L. represented the highest isolation rate (0.36) as against the lowest one in stem tissues of *H*. *annuus* L. (0.05). Colonizing frequency was also low in stem tissues (65.33%) compared to the root (98.63%).

Diversity of endogenous microbiota

The Shannon-Weaver diversity index showed that *B*. *nigra* L. harbour most diverse types of endogenous bacteria and more specifically, the leaves (16.35) produced highest number of phenotypically distinguishable bacterial strains than its stem (9.63) and root (10.51). Similarly, *B*. *nigra* L. leaves also showed highest Simpson (0.95) index. The Shannon-Weaver diversity index was recorded lowest in leaf tissues of *B*. *napus* L. (3.11), whereas lowest Simpson diversity index (0.63) was observed in leaf tissues of *H*. *annuus* L. (Table 1).

Screening of bacterial strains for P(3HB) production

All 154 phenotypically distinguishable bacterial isolates were screened for the accumulation of P(3HB) during growth under batch cultivation in glucose containing mineral salts medium. Intracellular accumulation of P(3HB) as determined by Nile blue A staining (Ostle and Holt, 1982) and chemical estimation (Law and Slepeckey, 1961) clearly indicated positive response by more than 77% of isolates with P(3HB) content ranging from 3.54 - 44.98% of cell dry weight (CDW). While, 96% of the isolates of *A*. *hypogaea* L. produced P(3HB), only 44% from *H*. *annuus* L. could have accumulated the polyester. Based on the amount of polyester produced, only 5 isolates representing the oleaginous species *A*. *hypogaea* L. (AHS 01, AHR 02), *B*. *napus* L. (BNL 103, BNS 102) and *R. communis* L. (RCL 02) were assigned as good (>35% CDW) P(3HB) producer (Table 2). These five

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Table 1: Diversity of microbiota endogenous to leaf, stem and root tissues of some selected oleaginous plants.

Parameters	Plant tissue	Oleaginous plant					
		Arachis hypogaea L.	*Brassica napus* L.	*Brassica nigra* L.	*Helianthus annuus* L.	*Ricinus communis* L.	*Sesamum indicum* L.
No. of segments used for isolation	Leaf	72	53	57	72	80	46
	Stem	79	52	63	75	80	44
	Root	68	53	68	73	80	43
Segments yielding bacterial isolates	Leaf	48	49	45	52	70	33
	Stem	59	48	43	49	64	38
	Root	61	50	50	72	68	29
Number of bacterial isolates	Leaf	05	08	21	08	07	06
	Stem	09	05	12	04	07	05
	Root	13	08	12	11	06	07
Colonizing frequency, %	Leaf	66.66	92.45	78.94	72.22	87.50	71.73
	Stem	74.68	92.31	68.25	65.33	80.00	86.36
	Root	89.70	94.34	73.52	98.63	85.00	67.44
Isolation rate	Leaf	0.07	0.15	0.36	0.11	0.09	0.13
	Stem	0.11	0.10	0.19	0.05	0.09	0.11
	Root	0.19	0.15	0.17	0.15	0.08	0.16
Shannon-Weaver index	Leaf	3.86	4.51	16.35	4.11	4.69	5.07
	Stem	5.80	3.11	9.63	3.71	5.79	4.22
	Root	8.79	3.89	10.51	7.04	4.31	5.98
Simpson index	Leaf	0.71	0.73	0.95	0.63	0.75	0.80
	Stem	0.81	0.64	0.91	0.73	0.81	0.75
	Root	0.87	0.67	0.90	0.84	0.73	0.84

Table 2: Screening of microbiota endogenous to selected oleaginous plants for production of poly(3-hydroxybutyrate).

Oleaginous plant	No. of bacterial isolate	No. of non-producer isolate	P(3HB) producing isolate[a]		
			Poor [<15%, CDW]	Moderate [15-35%, CDW]	Good [>35%, CDW]
Arachis hypogaea L.	27	01	19	05	02
Brassica napus L.	21	02	16	01	02
Brassica nigra L.	45	10	31	04	-
Helianthus annuus L.	23	13	06	04	-
Ricinus communis L.	20	03	13	03	01
Sesamum indicum L.	18	06	08	04	-
Total	154	35	93	21	05
Percentage (%)	100	22.72	60.39	13.64	3.24

[a], The isolates were grown in mineral salts medium and the intracellular accumulation of P(3HB) was determined by Nile blue A staining (Ostle and Holt, 1982) while, the polyester content of the dry cell mass was determined following the methods of Ramsay *et al.* (1990) and Law and Slepeckey (1961).

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isolates were selected for detailed characterization, identification and P(3HB) production.

Characterization and identification of bacterial isolates

Morphological and physio-biochemical characteristics of the selected P(3HB) producing isolates were determined following the standard protocols (Gerhardt *et al.*, 1994). Morphologically all of them were rod-shaped (Figure 1) and four of them were Gram-positive, while only AHR 02 was Gram-negative. The sporogenous isolates BNS 102 and RCL 02 produced all six hydrolytic enzymes tested, while AHR 02 was capable of producing only catalase, pectinase and cellulase. The pattern of sugar fermentation of BNS 102 and RCL 02 was more or less identical with the exception of cellobiose and turanose, while the other three showed distinct patterns of fermentation. Most of the isolates were resistant to ampicillin, methicillin, penicillin and to some extent chloramphenicol. Isolate AHR 02 was the most resistant one, while BNL 103 was resistant to only ampicillin and methicillin (Table 3).

The 16S rDNA sequence analysis revealed that AHS 01, AHR 02 and BNL 103 were most closely related to *Cellulosimicrobium cellulans* DSM 43879, *Beijerinckia fluminensis* UQM 1685 and *Exiguobacterium acetylicum* DSM 20416 respectively with a very high percentage of sequence similarity (99%), reasonably high score and E-value being zero. Moreover, BNS 102 showed 100% sequence homology with *Bacillus toyonensis* BCT-7112 while, RCL 02 seemed to be most closely (97%) related to *Bacillus cereus* ATCC 14579. The 16S rDNA sequences of these isolates have been deposited to the GenBank and designated as *Cellulosimicrobium cellulans* AHS 01 (KX458038), *Beijerinckia fluminensis* AHR 02 (KX458039), *Exiguobacterium acetylicum* BNL 103 (KX458037), *Bacillus toyonensis* BNS 102 (KX458036) and *Bacillus cereus* RCL 02 (KX458035). The evolutionary relationship of these isolates was depicted from the dendrogram that showed clear rooted evolution (Figure 2).

Effect of different media on P(3HB) production

The selected isolates were further tested in four different media in addition to mineral salts medium for determining the most suitable one for P(3HB) production. The bacterium, *B*. *cereus* RCL 02 showed maximum polyester accumulation (62.82%, CDW) at 48 h of incubation in yeast extract supplemented mineral salts medium. In fact, supplementation of yeast extract (0.2%) significantly enhanced both growth and P(3HB) production in all five isolates and appeared to be effective and suitable for polyester synthesis. Glucose casamino acid medium also influenced the growth and polyester accumulation positively. *B*. *toyonensis* BNS 102 and *C*. *Cellulans* AHS 01 produced maximum biomass (2.3 g/L) and P(3HB) (50.31%, CDW) in this medium. Davis and Mingoli's and tris-glucose media though apparently failed to provide sufficient nutritional support for growth and polyester production for most of the isolates, *B*. *fluminensis* AHR 02 could have accumulated 53.03% P(3HB) in tris-glucose medium after 72 h of incubation (Table 4). *B*. *cereus* RCL 02 also showed maximum P(3HB) yield (1.38 g/L) when grown in yeast extract supplemented mineral salts medium followed by *B*. *toyonensis* BNS 102 (1.12 g/L) and *B*. *fluminensis* AHR 02 (1.07 g/L) (data not shown).

FTIR spectral analysis

The overlaid FTIR spectra (Figure 3) of authentic P(3HB) and lyophilized whole cells of bacterial isolates *C*. *cellulans* AHS 01, *B*. *fluminensis* AHR 02, *E*. *acetylicum* BNL 103, *B*. *toyonensis* BNS 102 and *B*. *cereus* RCL 02 showed identical characteristic peak at 1720 cm^{-1} corresponding to ester carbonyl stretching (C=O). The peak at 3440 cm^{-1} represented the free O–H stretch of the polymer end groups. While the peaks at 2920-2980 cm^{-1} and 1240-1370 cm^{-1} represented the typical C–H bending of the aliphatic compounds, other minor peaks represented various cellular components.

^{1}HNMR spectral analysis

The solution state ^{1}HNMR spectra (at 300 MHz in a Bruker AV300) of the polyesters isolated and purified from the dry cell mass indicated chemical shifts at 1.2, 2.4-2.6 and 5.3 ppm corresponding to CH_3, CH_2 and CH groups respectively. The characteristic signals for other hydroxyalkanoic acids, however, were totally lacking and molecular composition as indicated by the chemical shifts (δ) confirmed the homopolymeric nature of the polyesters being composed solely of 3-hydroxybutyric acid (Figure 4).

DISCUSSION

Studies on the diversity of microbiota endogenous to oleaginous plants are essential in understanding their potential and importance in biotechnology. Bacterial endophytes colonizing the internal tissues of the oil-yielding plants *A*. *hypogaea* L. (Ibanez *et al.*, 2009), *B*. *napus* L. (Graner *et al.*, 2003) and *H*. *annuus* L. (Forchetti *et al.*, 2007) have been documented and explored for plant growth promotion, production of jasmonates, abscisic acid etc. However, reports on production of biopolyesters by endophytes of oleaginous plants are scanty (Das *et al.*, 2015; 2016). The present study represents a systematic survey of microbiota endogenous to some selected oleaginous plants for the production of biodegradable biopolyester, poly(3-hydroxybutyrate).

Among the oleaginous plants tested, *B*. *nigra* L. produced maximum number of phenotypically distinguishable bacterial isolates (45), while the diversity was comparatively low in *S*. *indicum* L. (18). The high colonizing frequency (98.63%), particularly of the root segments of *H*. *annuus* L. clearly revealed high population density of bacteria, although, the isolation rate was

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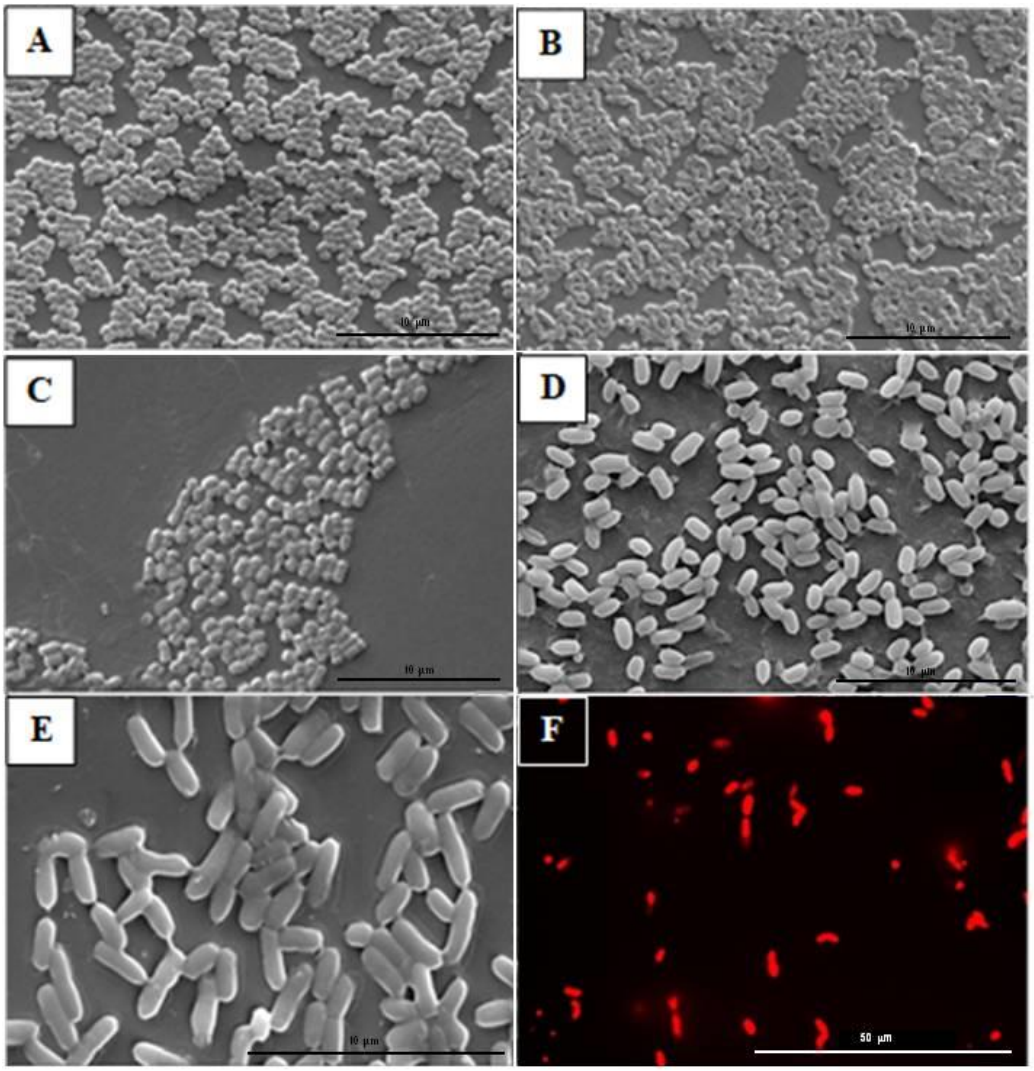

Figure 1: Scanning electron micrographs showing the morphology of P(3HB) producing bacteria endogenous to oleaginous plants. (A) *C*. *cellulans* AHS 01. (B) *B*. *fluminensis* AHR 02. (C) *E*. *acetylicum* BNL 103. (D) *B*. *toyonensis* BNS 102. (E) *B*. *cereus* RCL 02. (F) Photomicrograph showing the P(3HB) production by *B*. *cereus* RCL 02 cells following staining with 1% Nile blue A as revealed under fluorescence microscope.

highest (0.36) in *B*. *nigra* L. leaves reflecting that it harbours most diverse type of endogenous bacterial strains (21). Shannon-Weaver and Simpson indices which represent the number of distinguished phenotypes as well as evenness of distribution also support this observation (Table 1).

Screening of the endogenous microbial population (154) of selected oleaginous plants revealed predominance (77.27%) of bacteria capable of synthesizing and intracellularly accumulating P(3HB) during growth under batch cultivation (Table 2). These reserve biopolyesters could be of help in the survival and long term sustenance of these endospheric bacteria in the highly competitive and stressful microenvironment inside the plant. However, only five (3.24%) of the bacterial isolates accumulated substantial amount of P(3HB) which supports the earlier observations of P(3HB) production by the wild and mutant type of endophytic bacteria (Kamnev *et al.*, 2002; Catalan *et al.*, 2007; Das *et al.*, 2015).

Taxonomically, the apparently similar isolates, RCL 02 and BNS 102 were assigned to *Bacillus cereus* RCL 02 (GenBank accession number KX458035) and *Bacillus toyonensis* BNS 102 (GenBank accession number KX458036) respectively showing 97% and 100% sequence homology. Growth associated production of P(3HB) by members of the genus *Bacillus* is known to be influenced by various physico-chemical parameters (Wu *et al.*, 2001; Valappil *et al.*, 2007) and *B*. *cereus* alone has been reported to produce P(3HB) (Valappil *et al.*, 2007) along with P(3HB-co-3HV) co-polymers and terpolymers (Labuzek and Radecka, 2001). Likewise, based on very high sequence homology (99%) with NCBI library strains,

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Table 3: Morphological, physiological and biochemical characters of the selected bacterial isolates endogenous to oleaginous plants.

Characteristics	Bacterial isolates endogenous to oleaginous plants				
	AHS 01	AHR 02	BNL 103	BNS102	RCL 02
Colony morphology	Yellow, circular	Cream, circular	Yellowish orange, circular	White, irregular	White, rhizoidal
Cell morphology					
Shape	Rod	Rod	Rod	Rod	Rod
Arrangement	Single	Single	Single	Single/in pairs	Mostly in chains
Gram nature	Gram (+)	Gram (-)	Gram (+)	Gram (+)	Gram (+)
Endospore	-	-	-	Subterminal	Central
Motility	-	+/-	+	+	+
Diffusible pigment	Yellow	-	-	-	-
Enzyme production					
Catalase	+	+	+	+	+
Amylase	-	-	+	+	+
Lipase	-	-	-	+	+
Protease	+	-	+	+	+
Pectinase	+	+	+	+	+
Cellulase	+	+	+	+	+
Sugar fermentation					
Glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
Fructose	+	+	+	+	+
Maltose	+	+	+	+	+
Galactose	+	+	+	+	+
Lactose	-	-	-	-	-
Arabinose	-	+	-	-	-
Cellobiose	+	-	+	-	+
Turanose	-	+	-	+	-
Xylose	+	+	-	-	-
Mannitol	+	+	+	+	+
Sorbitol	-	-	-	-	-
Resistance to antibiotics	Amp, Chl, Kan, Met, Pen	Amp, Chl, Kan, Met, Pen, Van	Amp, Met	Amp, Met, Pen, Tet, Chl	Amp, Met, Pen

+, positive response; -, negative response. Antibiotics tested: Amp, ampicillin; Chl, chloramphenicol; Cip, ciprofloxacin; Ery, erythromycin; Gen, gentamicin; Kan, kanamycin; Met, methicillin; Pen, penicillin; Tet, tetracycline; Van, vancomycin.

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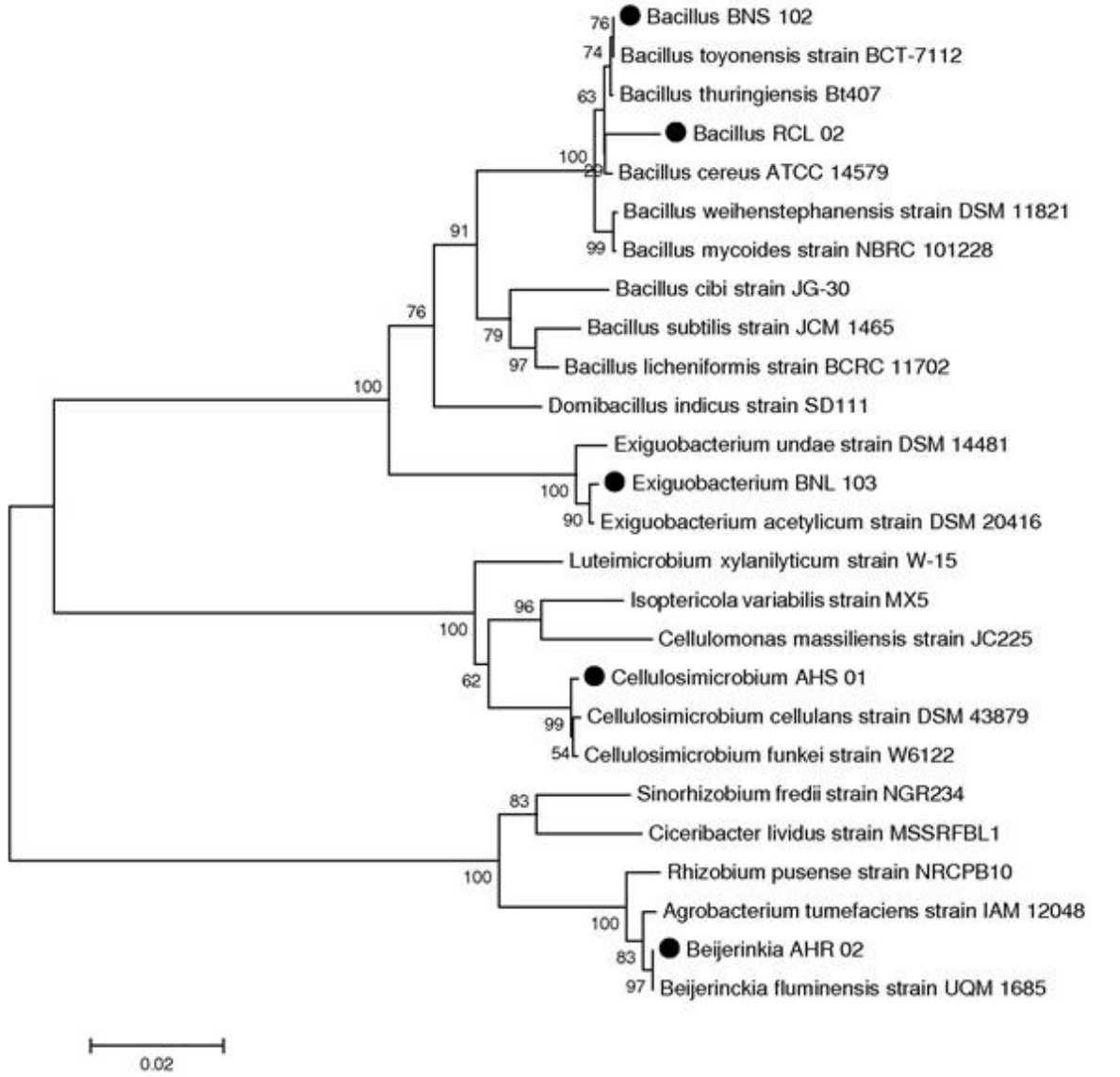

Figure 2: Phylogenetic relationship of the selected P(3HB) producing bacterial isolates with closely allied NCBI library strains based on 16S rDNA sequence analysis.

the isolates AHS 01, AHR 02 and BNL 103 have been identified and designated as *Cellulosimicrobium cellulans* AHS 01 (GenBank accession number KX458038), *Beijerinckia fluminensis* AHR 02 (GenBank accession number KX458039), and *Exiguobacterium acetylicum* BNL 103 (GenBank accession number KX458037) respectively (Figure 2). Endophytic existence of *Cellulosimicrobium* sp. in stems of medicinal plant *Maytenus austroyunnanensis* has been documented by Qin *et al.* (2012), but the production of P(3HB) by the endogenous bacterial isolate *C. cellulans* AHS 01 from *A. hypogaea* L. appears to be a new record. Likewise, Koomnok *et al.* (2007) in their studies have reported the existence of *Beijerinckia* sp. in the internal tissues of both cultivated and wild varieties of rice as well as their potential to fix atmospheric nitrogen. While, Stockdale *et al.* (1968) during the course of survey of members belonging to *Azotobacteriacea*, reported the occurrence of intracellular accumulation of P(3HB) by *Beijerinckia fluminensis*. Reports on endogenous association of *E. acetylicum* in the underground stem tubers of *Paris poliphylla chinensis* and production of diosgenin is well established (Ren *et al.*, 2007). Production of P(3HB) by *Exiguobacterium* sp. from diverse environmental samples are also not new (Kung *et al.*, 2007). As far as we are aware, this communication reports the endosphere

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Table 4: Influence of medium on growth and production of P(3HB) by selected bacterial isolates endogenous to some selected oleaginous plants.

Host plant	Tissue source	Bacterial isolate	Growth medium	Incubation, h			
				48		72	
				Growth, CDW, (g/L)	P(3HB), %, CDW	Growth, CDW, (g/L)	P(3HB), %, CDW
A. hypogaea L.	Stem	*Cellulosimicrobium cellulans* AHS 01	Davis Mingoli's medium	1.1 ± 0.08	25.98 ± 3.09	1.2 ± 0.11	31.62 ± 2.18
			Mineral salts medium	1.8 ± 0.28	35.28 ± 1.09	1.7 ± 0.09	32.19 ± 1.93
			Tris glucose medium	0.5 ± 0.12	10.12 ± 2.34	0.6 ± 0.15	19.78 ± 0.29
			Glucose-casamino acid medium	1.5 ± 0.19	42.83 ± 1.83	1.5 ± 0.21	50.31 ± 2.16
			Mineral salts medium + 0.2% YE	1.8 ± 0.23	43.13 ± 2.76	1.9 ± 0.17	48.63 ± 1.97
	Root	*Beijerinckia fluminensis* AHR 02	Davis Mingoli's medium	1.2 ± 0.19	14.37 ± 4.18	1.3 ± 0.03	17.82 ± 0.98
			Mineral salts medium	1.5 ± 0.17	42.89 ± 2.26	1.4 ± 0.12	42.96 ± 2.36
			Tris glucose medium	1.1 ± 0.06	52.42 ± 1.93	1.1 ± 0.04	53.03 ± 2.14
			Glucose-casamino acid medium	1.8 ± 0.18	42.12 ± 3.04	1.8 ± 0.09	43.27 ± 1.29
			Mineral salts medium + 0.2% YE	1.7 ± 0.02	55.32 ± 2.96	1.9 ± 0.07	56.34 ± 0.12
B. napus L.	Leaf	*Exiguobacterium acetylicum* BNL 103	Davis Mingoli's medium	1.1 ± 0.15	10.49 ± 0.98	0.9 ± 0.02	7.50 ± 1.02
			Mineral salts medium	1.3 ± 0.06	37.96 ± 1.04	1.2 ± 0.04	43.30 ±1.28
			Tris glucose medium	0.9 ± 0.05	19.78 ± 2.05	0.9 ± 0.08	17.16 ± 1.03
			Glucose-casamino acid medium	1.0 ± 0.21	30.31 ± 1.19	1.2 ± 0.12	35.32 ± 3.10
			Mineral salts medium + 0.2% YE	1.4 ± 0.19	50.30 ± 2.12	1.5 ± 0.05	52.50 ± 2.19
	Stem	*Bacillus toyonensis* BNS 102	Davis Mingoli's medium	1.6 ± 0.04	13.68 ± 4.03	1.3 ± 0.09	13.11 ± 0.04
			Mineral salts medium	1.9 ± 0.29	40.03 ± 2.67	1.8 ± 0.16	38.76 ± 0.84
			Tris glucose medium	1.6 ± 0.21	13.11 ± 1.32	1.6 ± 0.11	10.35 ± 1.35
			Glucose-casamino acid medium	2.3 ± 0.08	26.45 ± 0.29	2.2 ± 0.05	24.37 ± 2.06
			Mineral salts medium + 0.2% YE	2.0 ± 0.16	56.20 ± 3.97	2.0 ±0.17	55.10 ± 2.19
R. communis L.	Leaf	*Bacillus cereus* RCL 02	Davis Mingoli's medium	1.6 ± 0.27	15.29 ± 2.19	1.5 ± 0.08	13.39 ± 1.12
			Mineral salts medium	1.6 ± 0.12	44.98 ± 3.86	1.6 ± 0.16	38.27 ± 2.04
			Tris glucose medium	1.5 ± 0.17	18.86 ± 3.01	1.2 ± 0.06	12.85 ± 1.02
			Glucose-casamino acid medium	1.8 ± 0.18	42.88 ± 2.07	1.9 ± 0.13	37.78 ± 2.17
			Mineral salts medium + 0.2% YE	2.2 ± 0.17	62.82 ± 3.29	2.1 ± 0.13	60.74 ± 3.12

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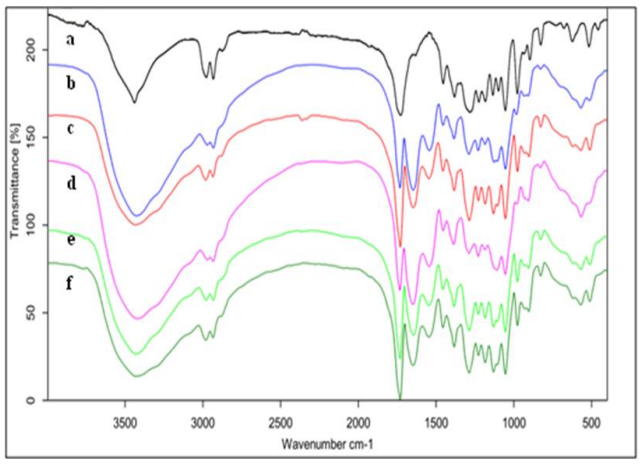

Figure 3: Fourier-transform infrared (FTIR) spectra of the purified P(3HB) (a), and lyophilized whole cells of *C*. *cellulans* AHS 01 (b), *B*. *fluminensis* AHR 02 (c), *E*. *acetylicum* BNL 103 (d), *B*. *toyonensis* BNS 102 (e), and *B*. *cereus* RCL 02 (f) harvested at late exponential phases of growth.

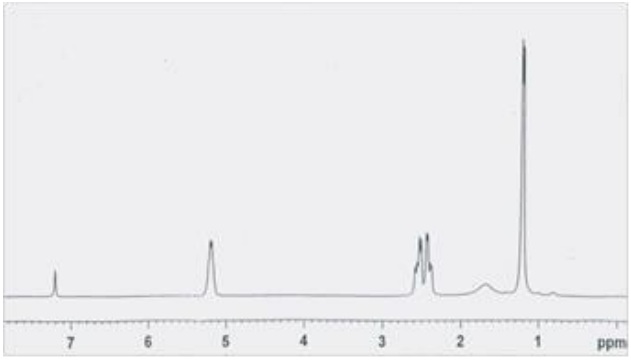

Figure 4: ^{1}HNMR spectrum of the biopolyester extracted from the representative bacterial isolate *B*. *toyonensis* BNS 102.

association as well as P(3HB) production by *E*. *acetylicum* from the oleaginous plant *B*. *napus.* Enhancement of growth and P(3HB) accumulation by the selected microbial strains have been accomplished by variation in culture media. Supplementation of yeast extract in mineral salts medium in general supported the biomass formation and P(3HB) production (Table 4). Likewise, amino acids present in glucose-casamino acid medium might have contributed to growth associated polymer accumulation. Further, the highest P(3HB) production (62.82%, CDW) by *B*. *cereus* RCL 02 also justifies the potential of endogenous microbiota of oleaginous plants in producing the biopolyester, poly(3-hydroxybutyrate). The FTIR spectra (Figure 3) of lyophilized whole cells of bacterial isolates showed characteristic peaks for –OH bending, C–H stretching , C=O carbonyl bonds and –CH aldehyde group in accordance with the confirmation of molecular composition of the accumulated polyesters to be P(3HB) (Hong *et al.*, 1999). Finally, the peaks in ^{1}HNMR spectra (Figure 4) of the purified polyesters were assignable to the methyl (CH_3; 1.2 ppm), methylene (CH_2; 2.4-2.6 ppm) and methine (CH; 5.2 ppm) carbon resonance of P(3HB) reported previously by Doi *et al.* (1989), which also provided supportive evidence in favour of intracellular P(3HB) accumulation by these endogenous bacteria of oleaginous plants.

CONCLUSION

Microbiota endogenous to higher plants have been proven to be rich resources of natural compounds of high level of structural diversity and wide spectrum of biological activities. This study has clearly demonstrated that bacteria endogenous to oleaginous plants are phenotypically quite distinguishable and appeared to be a potential source of biodegradable biopolyester, poly(3-hydroxybutyrate). Production of polyester by the selected isolates could further be enhanced in a prospective manner by optimization of cultural and nutritional conditions. These findings, therefore, deserve special attention to ascertain the viability of this endogenous microbial resources for large scale production of P(3HB) as an alternative to thermoplastics.

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