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Poly(3-hydroxybutyrate) Production By Seed Endophytic *Bacillus* spp. of Oleaginous Plants

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Abstract: *Seed endogenous microbiota is receiving significant interest as they are proven to be rich sources of novel natural compounds with wide spectrum of biological activities. In the present study, seed endophytes 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. were isolated and systematically screened for in vitro production and accumulation of biodegradable polyester poly(3-hydroxybutyrate) [P(3HB)]. Among the 45 phenotypically distinguishable bacterial endophytes isolated from surface sterilized seeds, nearly 77.8% of isolates showed significant amount of polyester accumulation in glucose containing mineral salts medium. The three most potent isolates were characterized and identified as Bacillus pumilus AHSD 04, Bacillus mycoides BNSD 10 and Bacillus cereus HASD 01 based on 16S rRNA gene sequence analysis and deposited to the GenBank under the accession numbers KY038573, KY029076 and KY029074, respectively. B. pumilus AHSD 04 and B. mycoides BNSD 10 accumulated P(3HB) accounting 70.3% and 53.3% of their cell dry weight (CDW) in glucose containing mineral salts medium supplemented with tryptose and tryptone, respectively. However, production was maximum (78.6%, CDW) in B. cereus HASD 01 when grown in sucrose and yeast extract supplemented medium. Spectroscopic (FTIR and ^{1}H NMR) analysis confirmed the identity of the intracellularly accumulated polyesters in all three endophytic isolates. This study indicates the possibility of exploring the oleaginous seed endophytic microbiota for production of the biopolyester, P(3HB) which has already established as an alternative to thermoplastics.*

Keywords: *Bacillus spp., oleaginous plant, poly(3-hydroxybutyrate), thermoplastics, seed endophytic bacteria*

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I. Introduction

Polyhydroxyalkanoates (PHAs), the aliphatic polyesters of 3-hydroxyacids are accumulated as intracellular inclusion in wide variety of bacteria inhabiting distinct ecological niches and serve as carbon and/or energy reserves [1]. Endophytic bacteria residing asymptomatically inside the internal tissues of plants are no exceptions and have received considerable attention for the production of biopolyesters [2]. These bacterial polyesters have been recognized as an alternative to conventional petrochemical based plastics due to their material properties, inherent biodegradability in nature and biocompatibility. Moreover, synthesis of PHA might be exhibited as one of the survival mechanisms of endosphere associated bacteria existing in a highly competitive and stressful microenvironment inside the plant.

Internal tissues of oleaginous plants which provide unique ecological habitat for growth and sustenance of endogenous bacteria have already been established as potential niche for PHA producers [3,4]. Seeds of the oleaginous plants contain considerable amount of lipids consisting primarily of triacylglycerols (TAGs) with varying composition of fatty acids depending on the plant species, seasonal variability and growth conditions. Recent studies have also investigated the feasibility of plant oils, as excellent carbon substrates for cost effective production of PHAs making the process economically viable [5].

Seed endophytes are of particular interest as they are considered to be transmitted from generation to generation through vertical transmission and assure their presence in progeny plants. They play significant role in plant growth promotion, phytoremediation and posses biocontrol activity. Members of the genus *Bacillus* have been recognized as the most abundant seed microbiota of diverse plant species where they are likely to play vital role in plant defence and growth stimulation [6]. Xu et al. [7] documented a seed borne *Bacillus subtilis* strain that improved the root and shoot growth of tomato by ACC deaminase production and nitrogen fixation. A study by Sobolev et al. [8] clearly revealed the prevalence of Gram-positive bacteria especially *Bacillus* spp. (94.4%) in seeds of *Arachis hypogaea* L. and most of them exhibited fungistatic and fungicidal

effect against *Aspergillus flavus*. However, no systematic studies have so far been conducted to explore the possibility of utilizing the seed endogenous microbiota for the synthesis and accumulation of PHAs.

The present study has focused attention on the accumulation of biopolyester poly(3-hydroxybutyrate) [P(3HB)], the most well studied PHA by seed endophytic *Bacillus* spp. isolated from the oleaginous plants *Arachis hypogaea* L., *Brassica napus* L., *Brassica nigra* L., *Helianthus annuus* L., *Ricinus communis* L. and *Sesamum indicum* L. under batch cultivation. Variation in different carbon and nitrogen sources has been explored to enhance the biopolyester production in three selected potent isolates and finally, the identity of the intracellularly accumulated polyesters was confirmed by spectroscopic analysis.

II. Materials And Methods

2.1. Collection of oleaginous plants and seeds

The 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) with mature pods were collected from Hooghly, Howrah and North 24-Parganas districts of West Bengal, India. The pods with mature seeds were thoroughly washed under running tap water and shelled aseptically for collection of seeds.

2.2. Isolation of seed endophytic bacteria

Endophytic bacteria were isolated from surface sterilized seeds following the technique of Hallmann et al. [9]. Seeds were sterilized by consecutive immersion in 70% ethanol (2-3 min) and 0.5% sodium hypochlorite (5-10 min) followed by rinsing for several times with sterile deionized water. Surface sterilized seeds were aseptically cut into segments, plated on glycerol asparagine agar, nutrient agar as well as tryptic soy agar media and incubated at 28-32 °C for 2-4 days. Growth of phenotypically distinguishable bacterial colonies adhering to the segments was observed and the bacterial cultures were obtained in pure form by dilution-streaking on same agar medium.

2.3. Characterization and identification of endophytes

The seed borne bacterial endophytes were characterized following standard morphological and physio-biochemical tests [10]. Susceptibility to different antibiotics was tested following Kirby Bauer disc diffusion method [11]. Phase contrast (Carl Zeiss No. 288997) and scanning electron (Zeiss Evo18 SEM) microscopy were used for determining cell morphology. The 16S rRNA gene sequences of the bacterial isolates were determined by direct sequencing of PCR amplified 16S rDNA. The genomic DNA of the freshly grown cell mass was isolated and purified according to the modified method of Marmur [12] and the 16S rDNA was amplified using the universal primers 27F (5′AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′GGTTACCTTGTTACGACTT3′). 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 neighbour-joining algorithm method with MEGA version 6.0 [13].

2.4. Screening of endophytes for P(3HB) production

For evaluation of P(3HB) production, the seed endophytic bacteria were grown in mineral salts medium [14] containing 2% (w/v) glucose as sole carbon source (25 mL per 100 mL Erlenmeyer flask) and incubated at 32 °C on a rotary shaker at 120 rpm. Samples were collected aseptically at definite time intervals and the cell mass was harvested by centrifugation (10,000 x g for 10 min). Dry weight of the washed cell mass was determined by drying to a constant weight at 80 °C.

The P(3HB) content of the dried biomass was extracted in warm chloroform [14] and recovered by precipitation with chilled diethylether. The dried polymer thus recovered was treated with H_2SO_4 in a boiling water bath for 10 min [15], 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 (CDW).

2.5. Spectroscopic analysis of polymer

2.5.1. FTIR spectral analysis

For Fourier transform infrared (FTIR) spectroscopy, the method of Kamnev et al. [16] was followed. Cells were harvested from the growing culture by centrifugation (10,000 x 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 Bruker FTIR spectrophotometer in the range of 4000 to 500 cm^{-1}.

2.5.2. ^{1}H NMR spectral analysis

The purified polyester samples 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 (300 MHz) 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 [17].

2.6. Statistical analysis

All the experiments were conducted in triplicate and the mean of the triplicate readings ± standard deviation for biomass and polyester production by the endophytic *Bacillus* spp. were recorded. One way analysis of variance (ANOVA) was implemented for evaluating the effects and interactions among different factors influencing the growth as well as P(3HB) accumulation. Tukey's adjustment was used to adjust the *p* values ($p < 0.05$) for multiple comparisons. All data management and analysis was done using Microsoft Excel and Origin Pro 8 software.

III. Results

3.1. Screening of seed endophytes for P(3HB) production

The phenotypically distinguishable 45 seed endophytic bacterial isolates obtained from surface sterilized seeds of *A. hypogaea* L. (9), *B. napus* L. (8), *B. nigra* L. (14), *H. annuus* L. (2) *R. communis* L. (5) and *S. indicum* L. (7) were screened for accumulation of P(3HB) during growth under batch cultivation. About 77.8% of these isolates showed P(3HB) production accounting 8.27-42.28% of CDW during growth (data not shown). Three of these seed isolates viz. AHSD 04, BNSD 10 and HASD 01 obtained from *A. hypogaea* L., *B. nigra* L. and *H. annuus* L. were selected as potent P(3HB) producers (>40%, CDW) for detailed characterization, identification and P(3HB) production in batch culture.

3.2. Characterization and identification of selected endophytes

Morphologically all three selected seed endophytes were Gram-positive, aerobic, rods (Fig. 1), produced sub-terminal to central endospores and tentatively identified as members of the genus *Bacillus*.

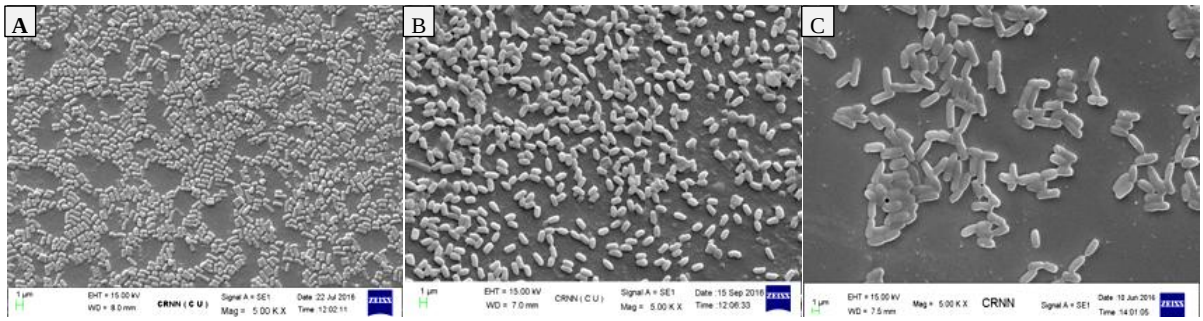

Fig.1: Scanning electron micrographs showing the morphology of P(3HB) producing seed endogenous bacterial isolates *B. pumilus* AHSD 04 (A), *B. mycoides* BNSD 10 (B) and *B. cereus* HASD 01 (C)

However, the isolates were distinguishable by their pattern of sugar fermentation and sensitivity to antibiotic (Table 1). The 16S rDNA sequence analysis revealed that isolates AHSD 04, BNSD 10 and HASD 01 were most closely related to *Bacillus pumilus* NBRC 12092, *Bacillus mycoides* ATCC 6462 and *Bacillus cereus* ATCC 14579, respectively with more than 99% sequence similarity, reasonably high score and e-value being zero. The 16S rDNA sequences of the isolates have been deposited to the GenBank and designated as *Bacillus pumilus* AHSD 04 (KY038573), *Bacillus mycoides* BNSD 10 (KY029076) and *Bacillus cereus* HASD 01 (KY029074). The evolutionary relationship of these isolates was depicted from the dendrogram that showed clear rooted evolution (Fig. 2).

Table 1 Phenotypically distinguishable characteristics of the selected seed endophytic *Bacillus* isolates

Characteristics	Seed endophytic bacterial isolates		
	B. pumilus AHSD 04	*B. mycoides* BNSD 10	*B. cereus* HASD 01
Colony morphology	Irregular, wrinkled, opaque	Rhizoidal	Dull, undulate margin
Cell size	2-3 µm × 0.6-0.7 µm	3-5 µm × 1-1.2 µm	3-5 µm × 1-1.2 µm
Motility	+	-	+
Hydrolysis of starch	-	+	+
Acid from:			
Arabinose	+	-	-
Galactose	+	-	-
Maltose	-	+	+
Mannose	+	+	-
Xylose	+	-	-
Mannitol	+	-	-
Resistance to antibiotic			
Ampicillin	-	+	+
Bacitracin	-	-	+
Ciprofloxacin	-	+	-
Erythromycin	-	+	+
Gentamycin	+	-	-
Novobiocin	+	-	-
Rifampicin	-	-	+
Tetracycline	-	+	+

"+" indicate positive response, "-" indicate negative response.

3.3. Effect of cultural conditions on P(3HB) production

3.3.1. Effect of media ingredients

The seed endophytes were screened for growth in five different media and results showed that supplementation of yeast extract (0.2%) in mineral salts medium significantly enhanced both growth and P(3HB) production. While, maximum accumulation of biopolyester (52.3%, CDW) by *B. pumilus* AHSD 04 was recorded at 72 h of incubation, *B. mycoides* BNSD 10 and *B. cereus* HASD 01 accumulated 56.9% and 58.9% P(3HB) of their cell dry weight respectively after 48 h of incubation under identical cultural conditions. However, maximum biomass (3.18 g/L) formation by *B. mycoides* BNSD 10 was recorded in glucose casamino acid medium (Table 2). Yeast extract supplemented mineral salts medium appeared to be most effective and suitable for polyester accumulation and therefore selected for further studies.

3.3.2. Growth Kinetics and P(3HB) production

Growth kinetics of seed endophytes conducted in yeast extract supplemented mineral salts medium under batch cultivation showed rapid increase of biomass accompanied by accumulation of P(3HB). While, in *B. mycoides* BNSD 10 and *B. cereus* HASD 01, maximum biomass formation (2.22 and 3.02 g/L respectively) was achieved after 56 h of incubation, growth in *B. pumilus* AHSD 04 was comparatively slow and reached a peak (2.15 g/L) after 72 h. In all three endophytic isolates growth was associated with rapid consumption of glucose and decline in pH (4.5-6.2) of the medium (data not shown). Rapid accumulation of P(3HB) was observed from early log phase and reached a maximum in the late stationary phase of growth. Maximum polyester production accounting 52.27%, 58.38% and 62.16% of cell dry weight were recorded in *B. pumilus* AHSD 04, *B. mycoides* BNSD 10 and *B. cereus* HASD 01 respectively. These seed endophytic *Bacillus* spp. started sporulating from late stationary phase and sporulation was accompanied by a sharp decline in the accumulated P(3HB) content of the cell mass (Fig. 3A-C).

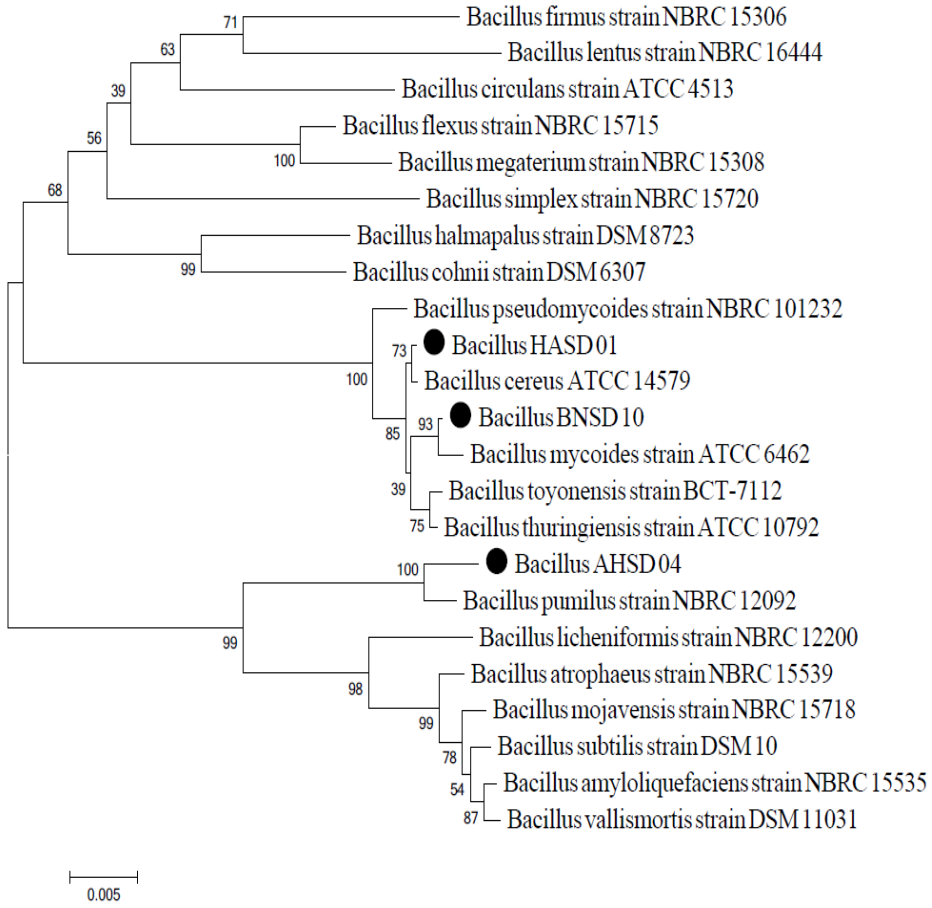

Fig. 2: Phylogenetic relationship of the potent P(3HB) producing *Bacillus* isolates with closely allied NCBI library strains based on 16S rDNA sequence analysis

3.3.3. Effect of carbon source

Among the three isolates, *B. mycoides* BNSD 10 and *B. cereus* HASD 01 efficiently utilized a number of different (12) carbon sources for growth and polymer production. But, P(3HB) accumulation by *B. pumilus* AHSD 04 was influenced only in the presence of glucose, sucrose, fructose, maltose, galactose, glycerol and mannitol in the medium (Table 3). Maximum biomass formation (2.15 g/L and 2.22 g/L) and polyester accumulation (52.3%, CDW and 58.4%, CDW) by *B. pumilus* AHSD 04 and *B. mycoides* BNSD 10 respectively were observed in glucose supplemented medium. Similarly, growth (3.12 g/L) and P(3HB) production (74.3%, CDW) by *B. cereus* HASD 01 were recorded in the presence of sucrose.

3.3.4. Effect of nitrogen source

Supplementation of both inorganic and organic nitrogen sources in the growth medium at 0.2% (w/v) level showed distinct variation in the growth and polymer production by the seed endogenous isolates. In comparison with inorganic nitrogen sources, organic nitrogenous compounds supported higher biomass and P(3HB) production. Highest growth (2.55 g/L) and polyester production (53.15%, CDW) by *B. pumilus* AHSD 04 was achieved by tryptose supplementation, while, tryptone significantly influenced the biomass formation (3.22 g/L) and P(3HB) accumulation (68.46%, CDW) in *B. mycoides* BNSD 10. However, yeast extract appeared to be the most suitable nitrogen source for growth (3.02 g/L) and biopolymer production (62.16%, CDW) by *B. cereus* HASD 01 (Table 4).

Table 2 Influence of culture media on growth and P(3HB) production by potent seed endophytic *Bacillus* isolates

Host plant	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	*B. pumilus* AHSD 04	A	0.45 ± 0.02	11.14 ± 0.76	0.25 ± 0.06	8.36 ± 0.08
		B	1.69 ± 0.02	41.27 ± 1.28	1.07 ± 0.02	40.02 ± 1.96
		C	0.49 ± 0.01	17.37 ± 1.42	0.84 ± 0.10	22.27 ± 0.98
		D	1.90 ± 0.08	43.56 ± 2.75	1.55 ± 0.07	34.29 ± 3.42
		E	1.96 ± 0.04	50.55 ± 0.68	2.15 ± 0.03	52.27 ± 0.34
		Comparison	F = 980.17	F = 370.13	F = 392.06	F = 256.50
			$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
B. nigra L.	*B. mycoides* BNSD 10	A	1.61 ± 0.12	13.30 ± 1.17	0.77 ± 0.01	16.78 ± 1.12
		B	1.20 ± 0.08	42.28 ± 2.36	1.13 ± 0.11	42.56 ± 2.16
		C	0.38 ± 0.01	31.76 ± 0.72	0.25 ± 0.02	42.76 ± 3.42
		D	3.18 ± 0.16	34.26 ± 2.17	2.78 ± 0.08	36.04 ± 0.85
		E	2.15 ± 0.07	56.88 ± 0.78	2.05 ± 0.14	52.75 ± 2.16
		Comparison	F = 320.52	F = 296.86	F = 400.17	F = 116.55
			$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
H. annuus L.	*B. cereus* HASD 01	A	1.39 ± 0.03	8.26 ± 0.15	1.11 ± 0.16	12.43 ± 0.64
		B	0.57 ± 0.05	40.19 ± 1.50	0.92 ± 0.01	38.28 ± 1.13
		C	0.27 ± 0.02	36.28 ± 1.38	0.28 ± 0.08	40.42 ± 2.56
		D	2.80 ± 0.21	53.03 ± 2.43	2.16 ± 0.02	54.00 ± 0.94
		E	2.85 ± 0.13	58.90 ± 0.95	2.36 ± 0.08	56.18 ± 0.18
		Comparison	F = 339.90	F = 527.82	F = 295.21	F = 499.95
			$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

* A = Davis Mingoli's medium, B = Mineral salts medium, C = Tris glucose medium, D = Glucose-casamino acid medium, E= Mineral salts medium + 0.2% YE.
Each value represents average of triplicate readings ± SD
p-values are adjusted for comparisons using Tukey's method; CDW= Cell dry weight

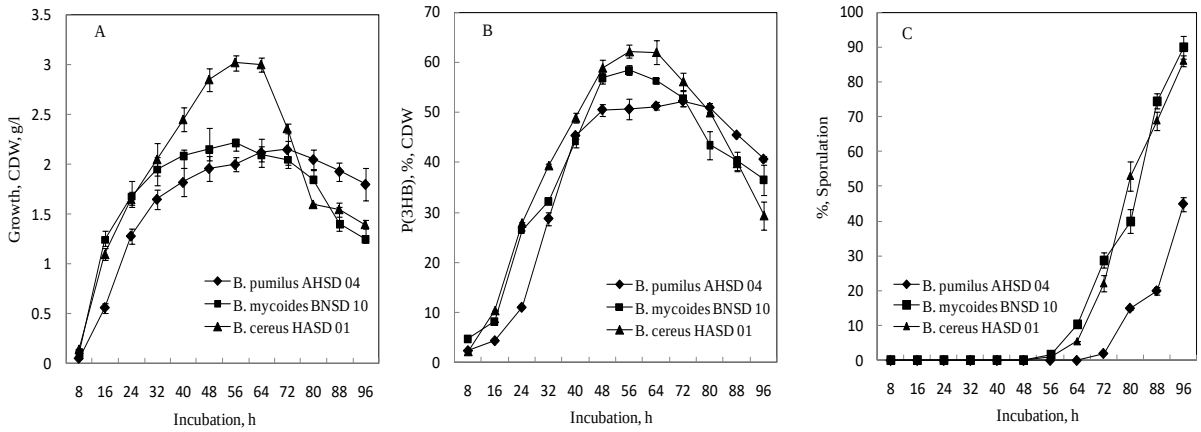

Fig. 3: Time course of growth (A), P(3HB) production (B) and degree of sporulation (C) of the seed endophytic *Bacillus* isolates *B. pumilus* AHSD 04, *B. mycoides* BNSD 10 and *B. cereus* HASD 01 in yeast extract supplemented mineral salts medium under batch cultivation

Table 3 Influence of carbon sources on growth and poly(3-hydroxybutyrate) production by potent seed endophytic *Bacillus* isolates

Carbon source, 2% (w/v)	Seed endophytes					
	B. pumilus AHSD 04		*B. mycoides* BNSD 10		*B. cereus* HASD 01	
	Growth, CDW(g/L)	P(3HB), % CDW	Growth, CDW(g/L)	P(3HB), % CDW	Growth, CDW (g/L)	P(3HB), % CDW
Glucose	2.15 ± 0.05	52.27 ± 0.85	2.22 ± 0.06	58.38 ± 1.11	3.02 ± 0.04	62.16 ± 1.78
Sucrose	0.79 ± 0.02	48.35 ± 2.12	0.91 ± 0.01	47.11 ± 2.23	3.12 ± 0.01	74.28 ± 1.14
Fructose	0.82 ± 0.11	28.74 ± 2.36	1.15 ± 0.02	20.34 ± 2.87	2.90 ± 0.00	36.70 ± 0.45
Maltose	0.43 ± 0.01	34.54 ± 3.02	1.77 ± 0.05	53.66 ± 3.93	3.05 ± 0.03	70.20 ± 2.29
Galactose	1.03 ± 0.04	25.36 ± 1.19	0.74 ± 0.14	23.56 ± 1.18	1.80 ± 0.03	46.00 ± 2.64
Lactose	1.27 ± 0.16	-	0.78 ± 0.11	28.76 ± 1.83	2.75 ± 0.06	20.85 ± 1.17
Glycerol	0.75 ± 0.08	18.48 ± 0.08	0.34 ± 0.01	50.29 ± 0.94	1.25 ± 0.01	39.80 ± 3.03
Mannitol	1.02 ± 0.02	34.67 ± 1.42	0.46 ± 0.04	28.43 ± 1.12	1.55 ± 0.09	57.43 ± 0.97
Na-propionate	1.07 ± 0.02	-	1.48 ± 0.03	24.38 ± 0.06	3.10 ± 0.12	72.12 ± 2.44
Na-citrate	1.03 ± 0.06	-	1.39 ± 0.02	20.30 ± 0.92	1.30 ± 0.06	16.98 ± 1.63
Na-acetate	0.98 ± 0.14	-	0.81 ± 0.09	18.45 ± 1.35	1.75 ± 0.02	60.65 ± 2.93
Hexane	0.35 ± 0.01	-	0.68 ± 0.02	10.18 ± 1.28	1.85 ± 0.01	17.75 ± 0.64
Comparison	F = 102.56	F =539.22	F = 225.52	F = 224.12	F = 617.14	F = 357.88
	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

Each value represents average of triplicate readings ± SD
p-values are adjusted for comparisons using Tukey's method
CDW = Cell dry weight
The isolates were grown in yeast extract (0.2%, w/v) supplemented mineral salts medium with different carbon sources (2%, w/v) for their respective optimum incubation time at 32 ºC under continuous shaking

3.3.5. Effect of C:N ratio

The best carbon and nitrogen sources which influenced maximum P(3HB) production were selected for all three isolates and C:N ratio was varied to study their influence on polymer production as well as growth. A glucose:tryptone, glucose:tryptose and sucrose:yeast extract at a ratio of 2:1 significantly influenced growth of *B. pumilus* AHSD 04 (2.77 g/L), *B. mycoides* BNSD 10 (3.72 g/L) and *B. cereus* HASD 01 (3.68 g/L) respectively under continuous shaking. However, P(3HB) accumulation was recorded maximum (53.3-78.6%, CDW) at C:N ratio of 5:1 in all three endophytic bacteria. The biopolyester production was recorded highest in *B. cereus* HASD 01 (78.6%, CDW) followed by *B. mycoides* BNSD 10 (70.3%, CDW) and *B. pumilus* AHSD 04 (53.3%, CDW) (Fig. 4).

3.4. Characterization of polymer

3.4.1. FTIR spectral analysis

The FTIR spectra (Fig. 5) of authentic P(3HB) and lyophilized whole cells of P(3HB) producing endophytic isolates *B. pumilus* AHSD 04, *B. mycoides* BNSD 10 and *B. cereus* HASD 01 showed characteristic peak at 1720 cm^{-1} corresponding to ester carbonyl stretching (C=O) of P(3HB). 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.

Table 4 Growth and poly(3-hydroxybutyrate) production by potent seed endophytic *Bacillus* isolates in various nitrogen sources

Nitrogen source, 0.2% (w/v)	Seed endophytes					
	B. pumilus AHSD 04		*B. mycoides* BNSD 10		*B. cereus* HASD 01	
	Growth, CDW(g/L)	P(3HB), % CDW	Growth, CDW (g/L)	P(3HB), % CDW	Growth, CDW(g/L)	P(3HB), % CDW
Bactopeptone	2.15 ± 0.08	48.98 ± 1.16	1.80 ± 0.02	34.20 ± 2.22	2.72 ± 0.01	60.46 ± 2.28
Beef extract	1.55 ± 0.02	46.48 ± 2.42	2.02 ± 0.12	18.15 ± 2.20	1.70 ± 0.03	40.16 ± 1.11
Casamino acid	1.70 ± 0.02	34.36 ± 3.33	2.10 ± 0.08	30.18 ± 0.05	2.15 ± 0.03	45.26 ± 0.89
Tryptone	2.25 ± 0.01	38.88 ± 1.93	3.22 ± 0.01	68.46 ± 1.16	2.65 ± 0.09	55.55 ± 2.67
Tryptose	2.55 ± 0.05	53.15 ± 1.02	3.15 ± 0.02	50.27 ± 3.28	2.92 ± 0.11	58.38 ± 3.15
Yeast extract	2.15 ± 0.05	52.27 ± 0.85	2.22 ± 0.06	58.38 ± 1.11	3.02 ± 0.04	62.16 ± 1.78
Ammonium chloride	1.20 ± 0.06	15.96 ± 0.08	1.26 ± 0.02	20.04 ± 2.14	1.55 ± 0.04	42.34 ± 0.38
Ammonium sulphate	1.07 ± 0.02	40.02 ± 1.96	1.15 ± 0.00	42.50 ± 0.64	0.86 ± 0.02	40.15 ± 0.66
Potassium nitrate	1.24 ± 0.04	29.76 ± 1.11	1.52 ± 0.09	23.34 ± 1.93	0.85 ± 0.00	40.10 ± 1.17
Sodium nitrate	1.95 ± 0.02	25.37 ± 0.94	1.52 ± 0.02	28.56 ± 2.36	0.95 ± 0.02	46.55 ± 2.92
Comparison	F = 420.28	F = 151.77	F = 452.28	F = 232.38	F = 871.98	F = 64.64
	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05

Each value represents average of triplicate readings ± SD
p-values are adjusted for comparisons using Tukey's method
CDW = Cell dry weight
The isolates were grown in glucose (2%, w/v) containing mineral salts medium with different nitrogen sources (0.2%, w/v) for their respective optimum incubation time at 32 ºC under continuous shaking

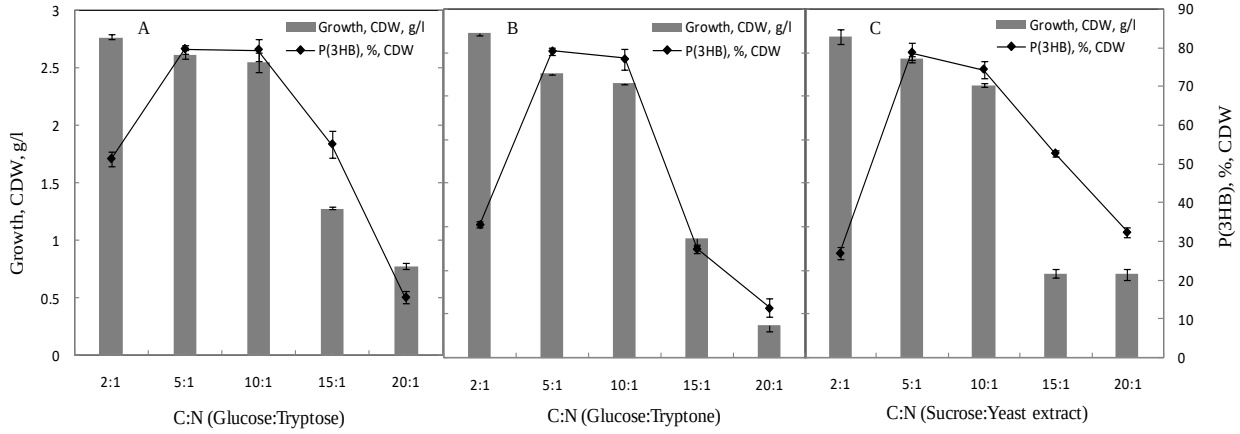

Fig. 4: Effect of C:N on growth and P(3HB) production by *B. pumilus* AHSD 04 (A) *B. mycoides* BNSD 10 (B) and *B. cereus* HASD 01 (C)

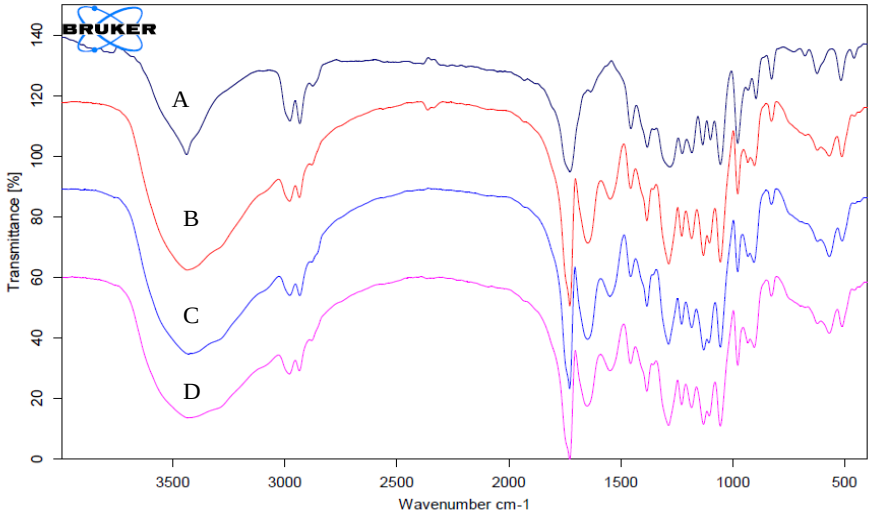

Fig. 5: Fourier-transform infrared (FTIR) spectra of the purified P(3HB) (A), and lyophilized whole cells of *B. pumilus* AHSD 04 (B), *B. mycoides* BNSD 10 (C) and *B. cereus* HASD 01 (D) harvested at late exponential phases of growth

3.4.2. ^{1}H NMR spectral analysis

The solution state ^{1}H NMR spectra (300 MHz in a Bruker AV300) of the polyesters isolated and purified from the dry cell mass of the seed endophytes indicated chemical shifts at 1.25, 2.42-2.63 and 5.21-5.27 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 (Fig. 6).

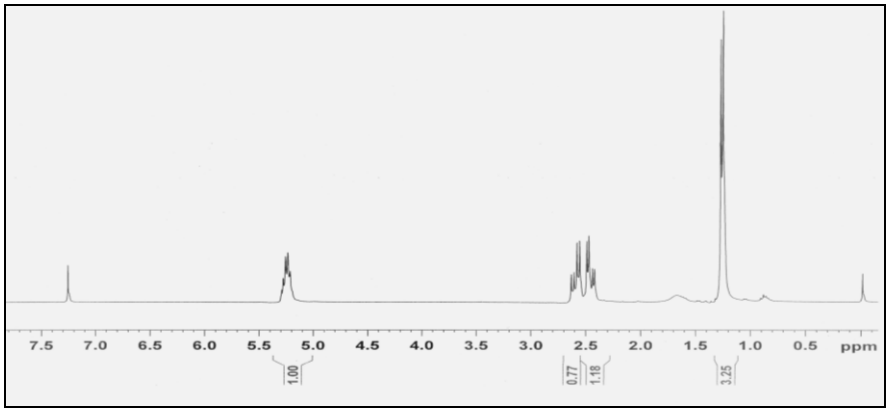

Fig. 6: ^{1}H NMR spectrum of the biopolyester extracted from the representative bacterial isolate *B. cereus* HASD 01 endophytic to seeds of *H. annuus* L.

IV. Discussion

Bacteria endogenously residing in seeds are known to play important role in germination, seedling development and plant growth [6]. In addition, seed endophytes often possess unique metabolic characteristics that can be explored for several biotechnological applications. Seeds of oleaginous plants characteristically synthesize and accumulate large amounts of lipoidal substances which are used for commercial purposes [18]. The present study represents a systematic survey of seed endophytic microbiota from *A. hypogaea* L., *B. napus* L., *B. nigra* L., *H. annuus* L., *R. communis* L. and *S. indicum* L. for the production of non-fossil fuel based biodegradable polyester poly(3-hydroxybutyrate) [P(3HB)]. Though the endosphere associated microbiota has been reported to produce biopolyesters [2,4], this report, as far as we are aware, is the first study to explore seed endophytic bacteria of oleaginous plants for the accumulation of P(3HB).

Screening of endophytic bacterial population of selected oleaginous seeds revealed that 77.8% of 45 isolates were capable of accumulating the intracellular biopolyester. However, only three isolates accumulating > 40% CDW P(3HB) were selected for detailed characterization and polyester production. The isolates were identified as *Bacillus pumilus* AHSD 04 (KY038573), *Bacillus mycoides* BNSD 10 (KY029076) and *Bacillus cereus* HASD 01 (KY029074) based on their morphological, physiological, biochemical characters and 16S rRNA gene sequence analysis (Table 1 and Fig. 2). These findings also confirm that *Bacillus* is the most predominant genus among the diverse seed endophytic bacterial genera reported so far [6]. Further, Sobolev et al. [8] have documented the occurrence of *B. thuringiensis*, *B. amyloliquifaciens*, *B. megaterium*, *B. cereus* and *B. subtilis* in the internal tissues of peanut seeds. The predominance of *Bacillus* spp. as seed colonizers may be attributed to the formation of endospores which protect them from adverse conditions prevailing inside the seeds [19,20].

Although, *Bacillus* spp. have long been found to produce P(3HB) [21,22] they are yet to be explored for commercial application because of the sporulation which is likely to interfere with the intracellular accumulation of biopolyester [23]. On the contrary, growth kinetics of *B. pumilus* AHSD 04, *B. mycoides* BNSD 10 and *B. cereus* HASD 01 showed suppression of sporogenesis during P(3HB) production (Fig. 3A-C) in exponential to early stationary phases of growth. This is in conformity with the previous studies of Kominek et al. [24] and Valappil et al. [25]. The delay in sporulation could be attributed to the presence of excess carbon as well as constituents of the medium which might have regulated the flux of metabolic intermediates towards growth associated P(3HB) production.

Influence of cultural conditions on growth and P(3HB) accumulation by potent seed endophytic isolates have shown that supplementation of yeast extract (0.2%, w/v) in mineral salts medium enhanced the biomass formation and P(3HB) production (Table 2). Amino acids present in glucose-casamino acid medium might have contributed to enhance growth (3.18 g/L) in *B. mycoides* BNSD 10. Utilization of variety of carbon sources for growth and P(3HB) production reflected variations in the carbon source utilization pattern by seed endophytes (Table 3). However, in comparison with inorganic nitrogen sources, complex organic nitrogenous compounds supported higher biomass formation and P(3HB) production (Table 4). Complex nitrogen sources such as, fish peptone, protease peptone, yeast extract, casitone, phytone and tryptone have already been documented to enhance P(3HB) production in *Azotobacter vinelandii* UWD [26]. In the present study, accumulation of P(3HB) by all three endophytic *Bacillus* spp. was triggered by an optimal C:N ratio of 5:1 (Fig. 4).

The overlaid FTIR spectra (Fig. 5) of polymer containing lyophilized whole cells of the 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 as P(3HB) [16] Finally, the peaks in ^{1}H NMR spectra (Fig. 6) of the purified polyesters were assignable to the methyl (CH_3; 1.25 ppm), methylene (CH_2; 2.42-2.63 ppm) and methine (CH; 5.21-5.27 ppm) carbon resonance of P(3HB) reported previously by Doi et al. [17], which also provided supportive evidence in favor of intracellular P(3HB) accumulation by these endophytic bacterial isolates of oleaginous seeds.

V. Conclusion

It is apparent that bacteria endogenous to seeds of oleaginous plants predominantly belonging to *Bacillus* spp. and are potential producers of biodegradable biopolyester, poly(3-hydroxybutyrate). The isolates are capable of producing considerable amount of biopolyester utilizing an array of carbon and nitrogen sources at specified C:N ratios. The present study, therefore, confirms the potential of seed endophytic *Bacillus* spp. to produce P(3HB) as an alternative to conventional thermoplastics for future biotechnological applications and a detailed physico-chemical analysis of accumulated polyester is essentially required for future exploitation of these seed endophytes.

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