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Influence of growth conditions on production of poly(3-hydroxybutyrate) by *Bacillus cereus* HAL 03 endophytic to *Helianthus annuus* L.

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ABSTRACT

Systematic screening of culturable endophytic bacteria for production of poly(3-hydroxybutyric acid) [P(3HB)] has resulted in the isolation of a potent strain identified as *Bacillus cereus* HAL 03 (GenBank Accession No. KR869088) from leaf tissue of *Helianthus annuus* L. Production of P(3HB) by this strain was influenced significantly by the quality as well as quantity of suitable carbon and nitrogen sources in the growth medium. At 2% (w/v) sucrose, the accumulated P(3HB) reached to a level of 50.46 % of its cell dry weight (CDW), whereas yeast extract (0.2%) as nitrogen source enhanced P(3HB) production and accounted for 53.19% of CDW. Moreover, the non-conventional carbon sources such as molasses further increased the production of the polyester up to 54.05% of its CDW. Finally, the identity of this intracellularly accumulated polyester was confirmed as a homopolymer of 3(hydroxybutyric acid) by the Fourier-transform infrared and proton nuclear magnetic resonance spectroscopic analysis. This study appears to indicate the first ever report of P(3HB) production by any strain of *B. cereus* endophytic to *H. annuus* L.

1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are linear biopolyesters of hydroxyalkanoic acids, which are biodegradable and biocompatible in nature and have the potential to act as bioplastics due to their physical and material properties analogous to synthetic plastics [1]. They are produced by a wide variety of microorganisms and are accumulated as intracellular inclusions or granules mostly under conditions of unbalanced growth [2]. To make the PHA bioplastic production economically viable and competitive with petroleum based thermoplastics, efforts are being made to find newer high-yielding microbial strains and also to develop low-cost production strategies.

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Plant offers a wide range of habitats that support microbial growth and the diversity of bacteria associated with the plants have been reported to accumulate PHAs including the most widely studied poly(3-hydroxybutyric acid) [P(3HB)]. While, the plant rhizosphere is an interesting hidden niche for polyhydroxyalkanoate producers [3, 4, 5], the culturable diversity of bacteria endophytic to plants have attracted the attention in recent years for the production of PHAs. Catalan *et al.* [6] have reported the accumulation of P(3HB) in the diazotrophic endophyte *Herbaspirillum seropedicae* Z69 colonizing a variety of higher plants. The bacterium accumulated 36% of its biomass as P(3HB) when grown in glucose containing medium. Comparative studies on the effects of heavy metals on endophytic and non-endophytic strains of *Azospirillum brasilense* [7], have revealed that non-endophytic strain of *A. brasilense* sp7 accumulated higher amount of P(3HB) than that of the endophytic strain of *A. brasilense* sp245 in response to heavy metals. Occurrence of endophytic bacteria in *Helianthus annuus* L., the important oil-yielding plant is not an exception and includes *Achromobacter xiloxidans*, *Alcaligenes* sp. and *Bacillus pumilus*, which so far have not been reported to synthesize and accumulate the polyester, P(3HB) [8].

Production of P(3HB) by members of the genus *Bacillus* has been reported extensively and found to be associated with their growth being influenced by various physico-chemical parameters. However, synthesis of P(3HB) by *Bacillus* spp. utilizing variety of readily available cost-effective substrates has been met with limited success for large scale production [9, 10]. Moreover, imbalance of nutritional conditions in *Bacillus* spp. have often been found to result in sporulation concomitant with reduction of P(3HB) production. Such a situation could be nullified by optimization of growth medium constituents as well as physical parameters. The ratio of carbon and nitrogen as well as the pH of the medium exert significant influence on the metabolism and accumulation of P(3HB) by *Bacillus cereus*. Synthesis and accumulation of P(3HB) by strains of *B. cereus* utilizing different carbon sources have been accounted for about 50% of CDW [10-19]. Moreover, production of co-polymers of 3-hydroxybutyric acid and 3-hydroxyvaleric acid, hydroxybutyric and hydroxycaproic acid and P(3HB) tercopolymers by strains of *B. cereus* are not uncommon [11, 13, 14].

In the course of our study of culturable endophytic bacteria isolated from *H. annuus* L. for production of poly(3-hydroxybutyrate), a leaf endophytic bacterium *B. cereus* HAL 03 most suitable for P(3HB) production has been selected, characterized in details and identified. Nutritional and cultural conditions including the utilization of non-conventional carbon sources by the isolate have been optimized for production of P(3HB) under batch cultivation and the identity of the polyester has been confirmed by spectroscopic analysis.

2. MATERIALS AND METHODS

2.1. Bacterial cultures and maintenance

The culturable endophytic bacterial strains used in this study were isolated from the surface sterilized leaf, stem and root segments of freshly collected *H. annuus* L. plants following the techniques of Hallmann *et al*. [20] and Reinhold-Hurek and Hurek [21]. Pure cultures of these isolates were maintained at 4ºC on slopes of tryptic soy agar by subculturing at regular interval.

2.2. Characterization of the bacterial isolate

The endophytic bacterial isolate was characterized and identified following standard morphological, physiological and biochemical tests [22] including susceptibility to different antibiotics [23]. The 16S rRNA gene sequence was determined by direct sequencing of PCR amplified 16S rDNA. The genomic DNA was isolated and purified according to the modified method of Marmur [24**]** and the 16S rDNA was amplified using the universal primers 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'GTTACCTTGTTACGACTT3'). 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 [25] with MEGA version 6.0.

2.3 Growth and P(3HB) production

Growth and P(3HB) production by bacterial endophytes were carried out in mineral salts medium [26]. The medium (50 ml / 250 ml Erlenmeyer flask) was inoculated with freshly prepared inoculum at 1% level and incubated on a rotary shaker (120 rpm) at 32ºC. Growth and P(3HB) content of the cell dry weight (CDW) were determined at regular time interval. While, growth of the isolates was estimated by the dry weight method, P(3HB) content of acetone dried cell mass was determined as per the method described by Law and Slepeckey [27]. Glucose in the medium was estimated following dinitrosalicylic acid method [28]. The percentage of sporulating cells was determined at regular time interval following examination of wet mounts of culture under phase contrast microscope (Carl Zeiss No. 288997).

2.4. FTIR spectral analysis

For Fourier transform infrared (FTIR) spectroscopy, the purified polyester isolated from the bacterial isolate HAL 03 was prepared as KBr pellet and scanned in a Perkin Elmer RX-1 FTIR spectrophotometer in the range of 4500 to 500 cm^{-1} [7, 29].

2.5. ^{1}H NMR analysis

Monomer composition of the purified polyester was determined by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. The polyester was dissolved in deuterochloroform ($CDCl_3$) and subjected to ^{1}H NMR analysis in a Bruker AV300 Supercon NMR spectrophotometer working in digital mode. A 5 nm BBO 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.

3. RESULTS AND DISCUSSION

3.1. Screening and selection of endophytic bacteria for P(3HB) production

Occurrence of endophytic bacteria with special reference to production of jasmonates and abscisic acid in culture has been well documented in *H. annuus* L. by Forchetti *et al*. [8]. The present study is an attempt to explore the potential of bacteria endophytic of *H. annuus* L. for the production of biodegradable biopolyester P(3HB). During the course of this study, a total of 23 phenotypically distinguishable endophytic bacterial isolates were obtained from the surface sterilized leaf, stem and root tissues of *H. annuus* L. and screened for the intracellular accumulation of P(3HB) during growth following chemical estimation method of Law and Slepeckey [27]. Growth associated P(3HB) accumulation was recorded in about 43.5% of the total endophytic isolates and

the P(3HB) content of these positive isolates ranged from 4.72-34.36% of their cell dry weight (CDW). These findings received support from the production of P(3HB) by endophytic diazotrophs [6, 7] as well as heterotrophs [30]. Out of these 23 isolates, bacterial strain HAL 03, endophytic to leaf of *H. annuus* L. appeared to be the most promising one showing the highest intracellular accumulation of polyester (34.36% of CDW) and was selected for detailed studies.

3.2. Characterization and identification of the strain HAL 03

Morphological, physiological and biochemical characteristics of the strain HAL 03 were determined following the standard protocol. The isolate HAL 03, a rod-shaped, motile, aerobic, Gram-positive, endospore forming bacterium was found to tolerate wide range of pH (3.5-8.0), temperature (25-42ºC) and NaCl (5-10%). It also produced a number of hydrolytic enzymes and showed characteristic pattern of sugar fermentation as well as sensitivity to a number of antibacterial antibiotics (Table 1). Based on these features, the strain HAL 03 was placed in the genus *Bacillus*. The 16S rDNA fragment of *Bacillus* HAL 03 was amplified by PCR using universal primers 8F and 1492R and a single discrete PCR amplicon band of approximately 1200 bp was resolved in an agarose gel. The PCR amplicon was further purified and sequenced in an ABI 377 automated DNA sequencer. The 16S rDNA sequence analysis revealed that the strain HAL 03 was most closely related to *Bacillus cereus* strain JCM 2152 with a very high sequence similarity (99%), reasonably high score and e-value being zero. Phylogenetically the strain showed close relationship with *Bacillus pseudomycoides*, *B. toyonensis* and *B. thuringiensis* along with a number of related species (Table 2). The evolutionary relationship as depicted from the dendrogram showed clear rooted evolution (Figure 1). The 16S rDNA sequence of the isolate HAL 03 has been deposited to the GenBank under the accession number KR869088 and designated as *Bacillus cereus* HAL 03.

Biosynthesis and characterization of P(3HB) by native strains of *B. cereus* have been investigated using variety of raw materials [9, 10]. Further efforts have also been made for the large scale production and efficient recovery of P(3HB) [10]. This study is probably the first ever report of the polyester production by any *B. cereus* strains endophytic to *H. annuus* L.

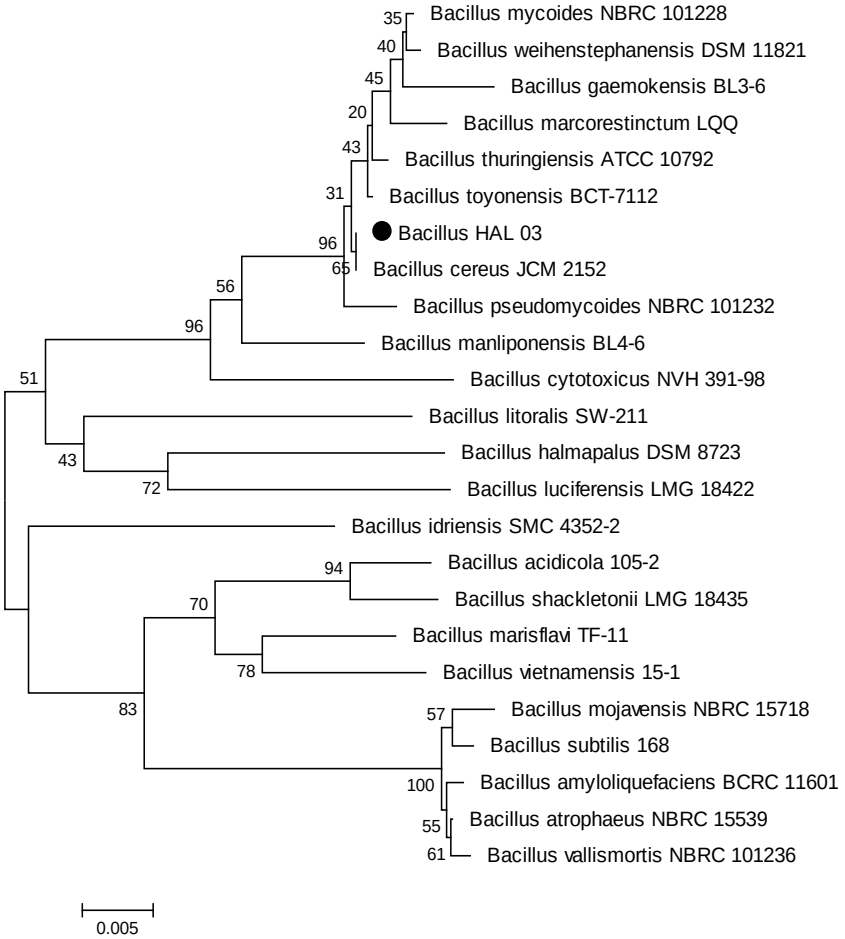

Fig. 1: Phylogenetic relationship of *Bacillus* HAL 03 (GenBank Accession Number KR869088) endophytic to *Helianthus annuus* L. with closely allied *Bacillus* spp. based on 16S rDNA sequence analysis.

Table 1: Morphological, physiological and biochemical characters of the bacterial strain HAL 03 endophytic to leaf tissues of *H. annuus* L.

Characters	Response
Morphological characteristics	
Colony morphology	White, round with smooth margin
Cell morphology	Rod-shaped, single , 2.75 µm×1.25µm
Gram reaction	Gram (+) positive
Endospore formation	+
Motility	+
Diffusible pigment production	None
Physiological characteristics	
Growth under aerobic condition	+
Growth under anaerobic condition	-
Growth on Mac Conkey agar	-
Range of pH for growth	3.5-8.0, optimum 7.6
Range of temperature for growth	25-42 ºC, optimum 37 ºC
NaCl tolerance	5-10 % (w/v)
Biochemical characteristics	
Production of catalase	+
Production of lipase	+
Production of cellulase	-
Production of pectinase	+
Hydrolysis of starch	+
Hydrolysis of casein	+
Liquefaction of gelatin	+
Reduction of nitrate	-
Production of acid from	Glucose, sucrose, fructose, maltose and galactose
Unable to ferment	Mannitol, lactose, xylose and sorbitol
Sensitive to antibiotics	Erythromycin, chlorotetracycline, vancomycin, methicillin, ciprofloxacin, kanamycin, gentamycin and penicillin
Resistant to antibiotics	Ampicillin and bacitracin

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

Morphology of the isolate was determined by growing on tryptic soy agar for 4 days at 32ºC. Fermentation of sugars was tested in phenol red agar medium supplemented with 1% carbon source. Antibiotic sensitivity of the isolates was determined by disc-diffusion assay [23].

Table 2: 16S rDNA sequence homology of *Bacillus cereus* HAL 03 (GenBank Accession Number KR869088) with closely related sequences from databases using BLAST analysis.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus cereus strain JCM 2152	1386	1386	100%	0.0	99%	NR113266.1
Bacillus pseudomycoides strain NBRC 101232	1371	1371	100%	0.0	99%	NR113991.1
Bacillus toyonensis strain BCT-7112	1368	1368	100%	0.0	99%	NR121761.1
Bacillus thuringiensis strain ATCC 10792	1368	1368	100%	0.0	99%	NR114581.1
Baciilus mycoides strain NRBC 101228	1362	1362	100%	0.0	99%	NR113990.1
Bacillus weihenstephanensis strain DSM 11821	1362	1362	100%	0.0	99%	NR024697.1
Bacillus cytotoxicus strain NVH 391-98	1287	1287	100%	0.0	97%	NR074914.1
Bacillus manliponensis strain BL4-6	1283	1283	95%	0.0	98%	NR125530.1
Bacillus gaemokensis strain BL3-6	1247	1247	91%	0.0	99%	NR116644.1
Bacillus marcorestinctum strain LQQ	1187	1187	87%	0.0	99%	NR117414.1
Bacillus litolaris strain SW-211	1178	1178	100%	0.0	94%	NR043015.1
Bacillus acidicola strain 105-2	1173	1173	100%	0.0	93%	NR041942.1
Bacillus halmapalus strain DSM 8723	1168	1168	100%	0.0	93%	NR026144.1
Bacillus luciferensis strain LMG 18422	1166	1166	100%	0.0	93%	NR025511.1
Bacillus marisflavi strain TF-11	1162	1162	100%	0.0	93%	NR025240.1
Bacillus shackletonii strain LMG 18435	1160	1160	100%	0.0	93%	NR025373.1
Bacillus atrophaeus strain NBRC 15539	1157	1157	100%	0.0	93%	NR112723.1
Bacillus valismortis strain NBRC 101236	1153	1153	100%	0.0	93%	NR113994.1
Bacillus amyloliquefaciens strain BCRC 11601	1153	1153	100%	0.0	93%	NR 116022.1
Bacillus idriensis strain SMC 4352-2	1153	1153	98%	0.0	93%	NR043268.1
Bacillus vietnamensis strain15-1	1153	1153	100%	0.0	93%	NR024808.1
Bacillus mojavensis strain NBRC 15718	1148	1148	100%	0.0	93%	NR112725.1
Bacillus subtilis strain 168	1142	1142	100%	0.0	93%	NR102783.1

3.3. Effect of different media on P(3HB) production

Four culture media of different composition were used to determine the most suitable one supporting growth and maximum P(3HB) accumulation by *B. cereus* HAL 03. Maximum biomass production (2.32 g/l) as well as P(3HB) accumulation (34.36%, CDW) were best supported by the components of mineral salts -medium as reported earlier by Ramsay *et al*. [26]. Likewise, amino acids present in glucose-casamino acid medium might have contributed to growth associated polymer accumulation. On the other hand, tris-glucose medium could not provide sufficient nutritional support for production of biomass as well as polyester by the endophytic strain (Figure 2).

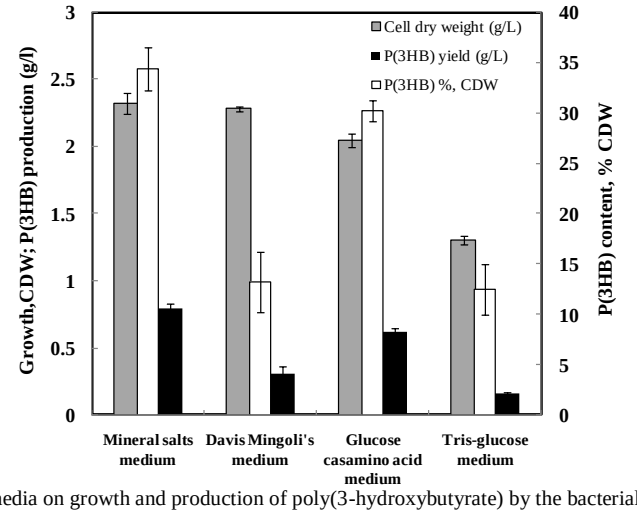

Fig. 2: Effect of different media on growth and production of poly(3-hydroxybutyrate) by the bacterial isolate *B. cereus* HAL 03

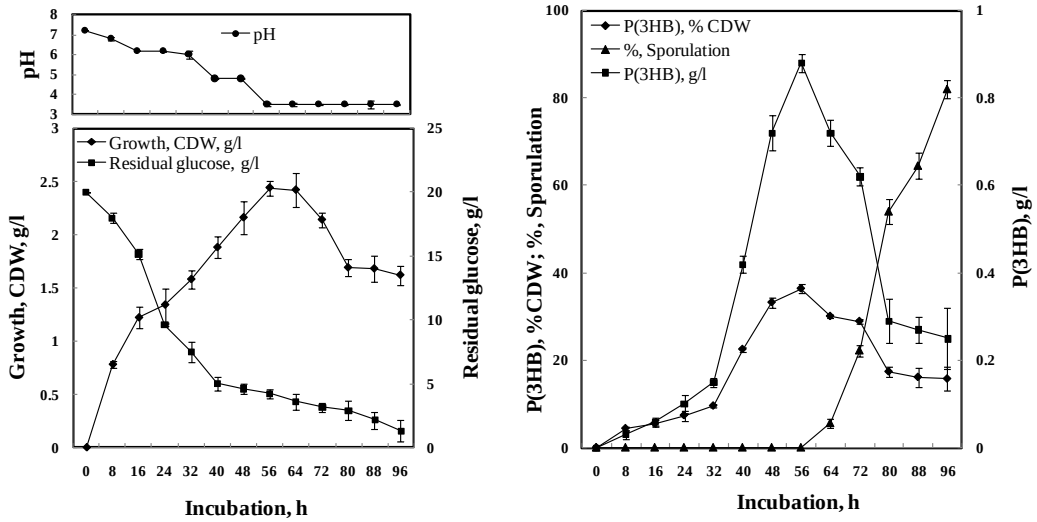

Fig. 3: Time course of growth, glucose utilization, changes in pH, poly(3-hydroxybutyrate) production, yield and sporulation by the bacterial isolate B. cereus HAL 03.

3.4. Time course of growth and P(3HB) accumulation

Time course of growth and P(3HB) accumulation by *B. cereus* HAL 03 showed that intracellular accumulation of P(3HB) was more or less parallel with growth and maximum P(3HB) accumulation (36.38%, CDW) was recorded after 56 h of incubation under continuous shaking (Figure 3). This is indicative of the fact that like many other heterotrophic bacteria, nutrient limitation is not an essential requirement for triggering P(3HB) production in *B. cereus* HAL 03. Furthermore, biosynthesis and accumulation of P(3HB) by HAL 03 was accompanied with the rapid utilization of glucose and as a consequence, the medium turned acidic (pH 3.5). Such decline in pH of the culture medium may possibly be due to the production of several acidic metabolic intermediates during growth. It has also been revealed that the accumulation of P(3HB) along with growth reached a maximum (56 h) just prior to the onset of sporulation, which received support from the earlier findings of Benoit *et al*. [31]. About 82% of cells showed mature spores at the end of 96 h of incubation. The formation of mature endospores in growing cells was associated with rapid decline of intracellular polymer indicating its possible utilization as carbon and energy source for sporogenesis. Delay in sporulation (initiation after 56 h) could be attributed to the presence of excess carbon as well as synthetic medium ingredients which probably regulated the flux of metabolic intermediates towards growth associated biosynthesis of P(3HB) by the present strain [32].

3.5. Effect of carbon source

The bacterium efficiently utilized glycerol, sucrose, maltose, fructose, mannitol, glucose and acetate for growth but higher polymer production and yield was observed in sucrose (50.46%, CDW) followed by fructose (46.44%, CDW) and glucose (34.36 %, CDW) (Table 3).

At an optimum concentration of sucrose (2%, w/v), the production and yield of P(3HB) were 50.83% and 1.37 g/l respectively. Further increase of sucrose in the medium was inhibitory to both growth and polymer accumulation (Figure 4).

Table 3: Growth and production of poly(3-hydroxybutyrate) by the endophytic bacterium *B. cereus* HAL 03 in various carbon sources.

Carbon source 2% (w/v)	Growth, CDW, g/l	P(3HB) production	
		P(3HB), % CDW	P(3HB), g/l
Glucose	2.32 ± 0.02	34.36 ± 2.2	0.79 ± 0.04
Fructose	2.53 ± 0.07	46.44 ± 1.7	1.17 ± 0.11
Sucrose	2.65 ± 0.14	50.46 ± 3.1	1.34 ± 0.12
Maltose	2.65 ± 0.11	26.74 ± 0.3	0.70 ± 0.03
Galactose	0.75 ± 0.09	29.76 ± 0.5	0.22 ± 0.05
Mannitol	2.53 ± 0.05	27.32 ± 1.2	0.69 ± 0.06
Glycerol	3.53 ± 0.07	23.58 ± 1.4	0.83 ± 0.09
Sodium acetate	2.25 ± 0.16	09.37 ± 0.3	0.21 ± 0.05
Sodium citrate	1.73 ± 0.05	12.59 ± 0.8	0.22 ± 0.04
Sodium gluconate	0.88 ± 0.08	08.57 ± 1.6	0.07 ± 0.12

Each value represents average of triplicate readings ±SE; CDW = Cell dry weight. The isolate, *B. cereus* HAL 03 was grown in mineral salts medium supplemented with different carbon sources (2%, w/v) for 56 h at 32ºC under continuous shaking. Growth was measured by cell dry weight and the P(3HB) was extracted with chloroform from acetone dried cell mass and quantified according to Law and Slepeckey [27].

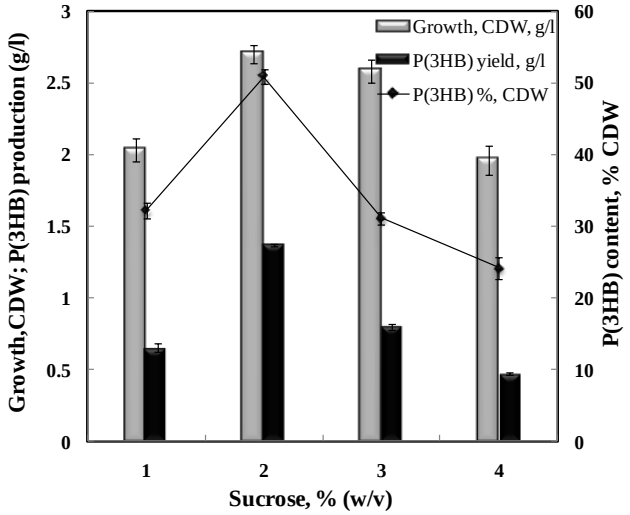

Fig. 4: Effect of sucrose concentrations on growth and production of poly(3-hydroxybutyrate) by the bacterial isolate *B. cereus* HAL 03.

Table 4: Growth and production of poly(3-hydroxybutyrate) by the endophytic bacterium *B. cereus* HAL 03 in various nitrogen sources.

Nitrogen source 0.1% (w/v)	Growth, CDW, g/l	P(3HB) production	
		P(3HB), % CDW	P(3HB), g/l
Beef extract	1.75 ± 0.04	43.70 ± 1.23	0.76 ± 0.04
Bactopeptone	1.80 ± 0.08	31.34 ± 1.89	0.56 ± 0.15
Yeast extract	1.98 ± 0.12	48.83 ± 2.01	0.96 ± 0.25
Casamino acid	1.25 ± 0.03	18.52 ± 2.07	0.23 ± 0.06
Tryptone	1.68 ± 0.03	19.55 ± 0.34	0.33 ± 0.01
Ammonium sulfate	2.32 ± 0.02	34.36 ± 2.2	0.79 ± 0.04
Ammonium nitrate	0.92 ± 0.10	23.86 ± 1.19	0.22 ± 0.12
Ammonium chloride	1.35 ± 0.12	7.46 ± 1.34	0.10 ± 0.16
Sodium nitrate	1.23 ± 0.02	27.31 ± 0.85	0.34 ± 0.01

Each value represents average of triplicate readings ±SE; CDW = Cell dry weight. The isolate, *B. cereus* HAL 03 was grown in mineral salts medium supplemented with different nitrogen sources (0.1%, w/v) for 56 h at 32ºC under continuous shaking. Growth was measured by cell dry weight and the P(3HB) was extracted with chloroform from acetone dried cell mass and quantified according to Law and Slepeckey [27].

3.6. Effect of nitrogen source

Among the different inorganic and organic nitrogen sources tested, ammonium sulfate resulted in significant increase in biomass formation, while yeast extract promoted the production of P(3HB) (Table 4). Further increase of yeast extract in the medium resulted enhanced growth and polyester production and maximum P(3HB) accumulation (53.2%, CDW) was recorded at 0.2% yeast extract (Figure 5). However, the yield of P(3HB) in ammonium sulfate (0.79 g/l) was comparable with that in beef extract (0.76 g/l).

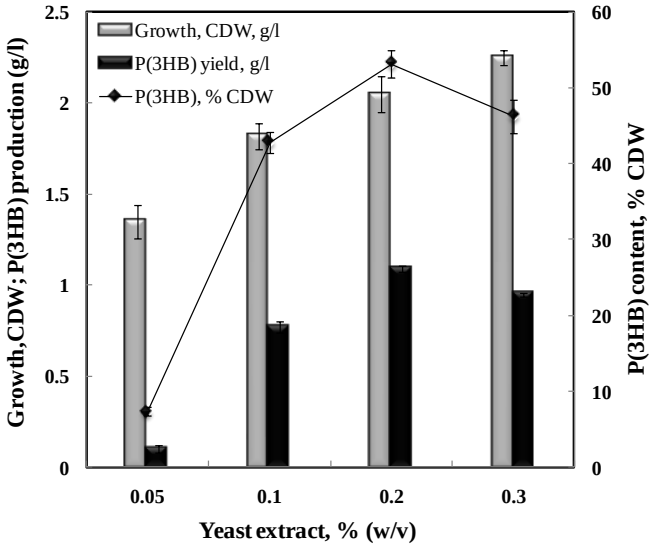

Fig. 5: Effect of different concentrations of yeast extract on growth and production of poly(3-hydroxybutyrate) by the bacterial isolate *B. cereus* HAL 03.

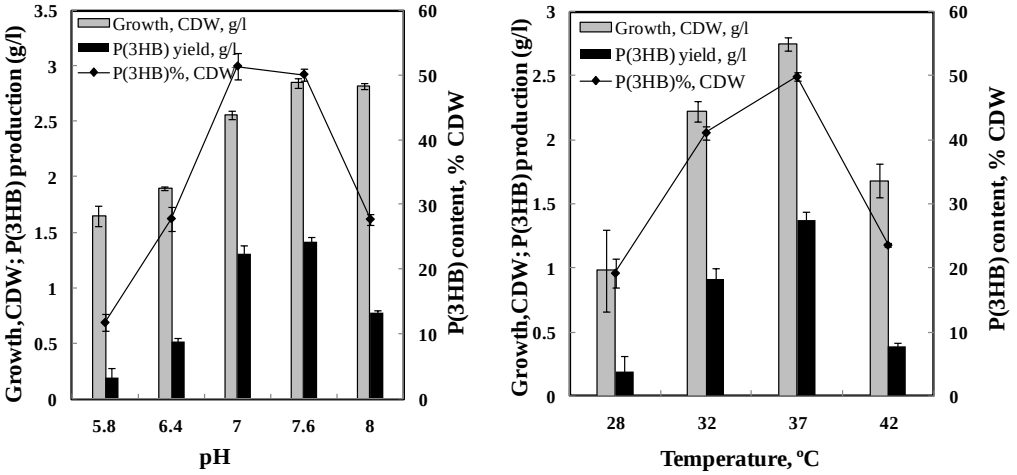

Fig. 6: Influence of pH and temperature on growth and poly(3-hydroxybutyrate) production by the bacterial isolate *B. cereus* HAL 03.

3.7. Effect of pH and temperature

The optimum pH and temperature for polymer accumulation coincide with those of growth of *B. cereus* HAL 03. The highest growth and P(3HB) accumulation were recorded at pH 7.6 and at a temperature of 37°C respectively (Figure 6). These findings confirmed the neutrophilic and mesophilic nature of the organism.

3.8. Effect of aeration

The effect of aeration on growth and P(3HB) production was determined under shaking conditions with variation of culture volume per flask volume (CVF). Increase of CVF (which decreases the rate of aeration of the culture) in sucrose containing medium resulted in increase of biomass but significantly reduced the polymer accumulation (Figure 7). Growth of the organism was maximum at a CVF of 4:10 (40 ml medium/100 ml flask), while, highest P(3HB) accumulation was recorded at a CVF of 2.5:10 (25 ml medium/100 ml flask). The increase in biomass at high CVF indicated efficient growth of the strain under oxygen stressed condition but sufficient supply of oxygen appeared to be an essential requirement for polyester synthesis and accumulation by HAL 03.

3.9. Effect of non-conventional carbon sources

Supplementation of growth medium with molasses of different types and malt extract (2%, w/v) as sole source of carbon were tested for growth and accumulation of the polyester by *B. cereus* HAL 03. Light molasses significantly influenced the biomass formation (2.75 g/l) and the production of P(3HB) reached up to 1.49 g/l with P(3HB) content of 54% of CDW (Table 5). Sugarcane molasses, the by-products of sugar industry, therefore, could serve as the cheap non-conventional source of carbon along with vitamins and other minor constituents for effective and sustainable production of P(3HB) by *B. cereus* HAL 03 similar to those of *Alcaligenes eutrophus* [33], *Bacillus megaterium* [34] and *Bacillus* sp. JMa5 [9].

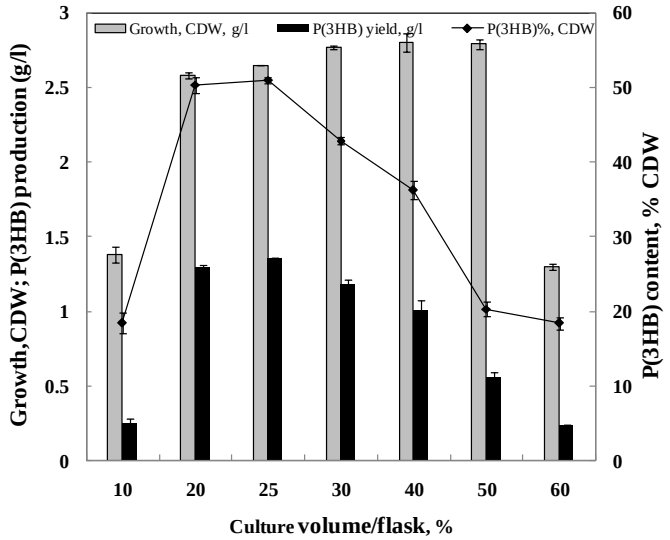

Fig. 7: Effect of aeration on growth and poly(3-hydroxybutyrate) production by the bacterial isolate *B. cereus* HAL 03

Table 5: Growth and production of poly(3-hydroxybutyrate) by the endophytic bacterium *B. cereus* HAL 03 using non-conventional carbon sources.

Non-conventional carbon source	Growth, CDW, g/l	P(3HB) production	
		P(3HB), % CDW	P(3HB), g/l
Purified molasses	1.42 ± 0.09	51.02 ± 2.04	0.72 ± 0.18
Light molasses	2.75 ± 0.32	54.05 ± 1.06	1.49 ± 0.33
Dark molasses	2.40 ± 0.08	43.41 ± 1.79	1.04 ± 0.14
Black strap molasses	1.75 ± 0.26	48.30 ± 0.93	0.84 ± 0.24
Malt extract	2.28 ± 0.43	39.67 ± 1.29	0.90 ± 0.15

Each value represents average of triplicate readings ±SE; CDW = Cell dry weight. The isolate, *B. cereus* HAL 03 was grown in mineral salts medium supplemented with different non-conventional carbon sources (2%, w/v) for 56 h at 32ºC under continuous shaking. Growth was measured by cell dry weight and the P(3HB) was extracted with chloroform from acetone dried cell mass and quantified according to Law and Slepeckey [27].

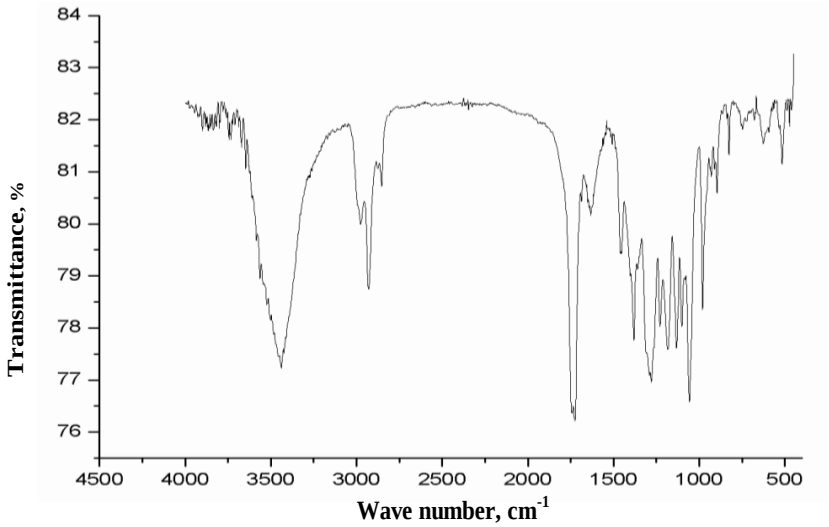

Fig. 8: Fourier-transform infrared (FTIR) spectrum of the polymer extracted from the bacterial isolate *B. cereus* HAL 03

3.10. FTIR and ^{1}H NMR spectral analysis

The identity of the intracellularly accumulated P(3HB) was further confirmed by Fourier-transform infrared (FTIR) and proton nuclear magnetic resonance (^{1}H NMR) spectral analysis. The FTIR spectra of the purified polyester (Figure 8) showed characteristic –OH 'bending at 3440 cm-1, C-H stretching at 2920-2980 cm^{-1}, C=O carbonyl bonds at 1720 cm^{-1} and –CH group of aldehyde at 1240-1370 cm^{-1} and provided evidence for the accumulation of P(3HB) by the endophytic bacterium *B. cereus* HAL 03 [7, 21]. Likewise, the solution state ^{1}H NMR spectrum of the polymer (Figure 9) indicated chemical shifts at 1.2, 2.4-2.6 and 5.3 ppm corresponding to CH_3, CH_2 and CH groups respectively characteristics of 3-hydroxybutyric acid and thereby confirmed the homopolymeric nature of the polyester being composed solely of 3-hydroxybutyric acid [35].

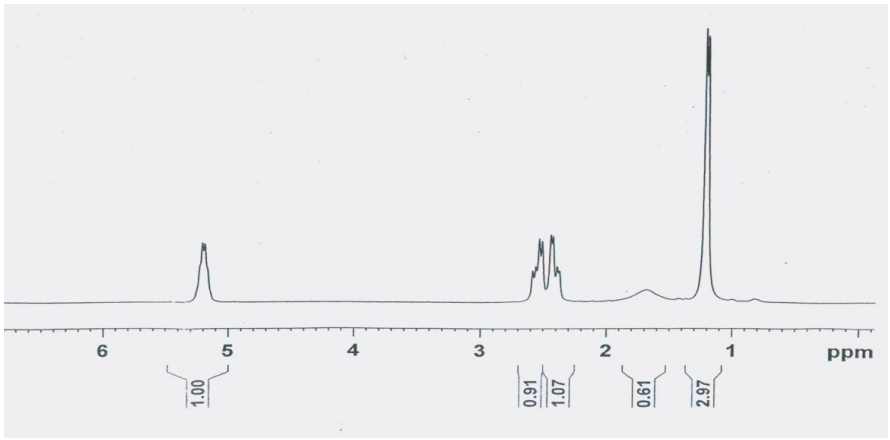

Fig. 9: Proton nuclear magnetic resonance spectrum of the polymer extracted from the bacterial isolate *B. cereus* HAL 03.

4. CONCLUSION

Endophytes have been proven to be rich sources of novel natural compounds with wide spectrum of biological activities and high level of structural diversity. This study has clearly demonstrated that *B. cereus* HAL 03, endophytic to leaves of *H. annuus* L. could synthesize and accumulate biodegradable biopolyester, P(3HB) and represents the first report of this kind. Production of P(3HB) by the strain was further enhanced by optimization of cultural conditions and a considerable enhancement was recorded by using cost effective non-conventional carbon sources. These findings, therefore, deserve more detailed experimentation and analysis to ascertain the viability of this endophytic microbial resource for large scale production of poly(3-hydroxybutyrate) as an alternative to thermoplastics.

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