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Research Article

Rapid Identification of Polyhydroxyalkanoate Accumulating Members of Bacillales Using Internal Primers for *phaC* Gene of *Bacillus megaterium*

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Bacillus megaterium is gaining recognition as an experimental model and biotechnologically important microorganism. Recently, descriptions of new strains of *B. megaterium* and closely related species isolated from diverse habitats have increased. Therefore, its identification requires several tests in combination which is usually time consuming and difficult to do. We propose using the uniqueness of the *polyhydroxyalkanoate synthase C* gene of *B. megaterium* in designing primers that amplify the 0.9 kb region of the *phaC* for its identification. The PCR method was optimized to amplify 0.9 kb region of *phaC* gene. After optimization of the PCR reaction, two methods were investigated in detail. Method I gave an amplification of a single band of 0.9 kb only in *B. megaterium* and was demonstrated by several strains of *B. megaterium* isolated from different habitats. The use of Method I did not result in the amplification of the *phaC* gene with other members of Bacillales. The specificity for identification of *B. megaterium* was confirmed using sequencing of amplicon and RT-PCR. Method II showed multiple banding patterns of nonspecific amplicons among polyhydroxyalkanoate accumulating members of Bacillales unique to the respective species. These methods are rapid and specific for the identification of polyhydroxyalkanoate accumulating *B. megaterium* and members of Bacillales.

1. Introduction

Bacillus megaterium is a Gram-positive, aerobic, spore forming bacterium present in diverse habitats from terrestrial to marine sediments. This "big beast" has been identified as an experimental organism for studies on various cell structures and functions [1, 2]. Its ecological and economical value has been established and reviewed [3–5]. It is only in the past two decades that various new strains of this species with immense potential in industries have been isolated from various ecosystems. The presence of *B. megaterium* in soil has been implicated in the degradation of herbicides and insecticides [6, 7]. Strains of *B. megaterium* have been recognised for biological control of root infections due to *Rhizoctonia solani* [8]. Strains producing biomolecules, such as phytohormone, 2-pentylfuran, pyruvate, oxetanocin, vitamin B12, and polyhydroxyalkanoate (PHA) which are of industrial importance have been investigated [9–12]. This bacterium produces proteins of economical importance, namely, agarase, chitosanase, penicillin G acylase, amylases, glucose dehydrogenase, neutral protease, and the family of P-450 cytochrome monooxygenases [13–15]. *Bacillus megaterium* is also amenable for recombinant DNA technology for the improvement of specific enzymes and for protein production [5]. Genomes of biotechnologically important strains of *B. megaterium* have been sequenced in 2011 [16]. Recently, it has been recognised as a promising candidate among Gram-positive organisms for producing polyhydroxyalkanoate on an industrial scale because few strains are capable of accumulating the homopolymer and copolymer in the presence of a single carbon source or inexpensive carbon substrates [17–19].

Phenotypic characterization for identification of strains of *B. megaterium* is based on morphology, nutrition, growth, and physiological characteristics including utilisation of various substrates [20–25]. Large variations and inconsistencies in test results make the identification of this species difficult. In the light of this, several methods have been employed for identification of strains of *B. megaterium* such as DNA-DNA hybridization [26], analysis of cellular fatty acid content [27], small-subunit-ribosomal RNA sequencing [20, 28], 16S rDNA sequencing [29], randomly amplified polymorphic DNA (RAPD) techniques [30, 31], SDS-PAGE of whole cell protein profiles [32], and PHA inclusion body associated protein profiles [27]. However, these methods are laborious, time consuming and have to be used in combinations.

In the last decade, several new members of *Bacillus* closely related to *Bacillus megaterium* have been described. On account of limited differences within closely related species, it is often problematic to differentiate between *B. megaterium* and its closely related *Bacillus* species using phenotypic characteristics and 16S rRNA sequences. This has been further compounded due to the establishment of new genera and groups within *Bacillus* that has led to the creation of new genera and realignment of several species [33–38]. Lately, many new species in Bacillales have been described. It requires a large number of tests to be performed with caution including RAPD analysis and SDS-PAGE profiling of whole cell proteins or polyhydroxyalkanoate inclusion body associated proteins to identify *B. megaterium*. The present report describes a simple and rapid method for identification of *B. megaterium* using the gene of PHA synthase C (*phaC*). Earlier this gene was thought to belong to Class III PHA synthase [39]. Recently, McCool and Cannon [2] reported it as being novel and distinctly different from other known PHA synthases. In the present study amplification of internal portion of *phaC* gene was devised as a novel, rapid, and simple technique for identification of *Bacillus megaterium*. Further, the variant of the method can be used in identifying PHA accumulating members of Bacillales.

2. Materials and Methods

2.1. Bacterial Strains and Growth Medium. Bacterial cultures used in the present investigation and their sources are listed in Table 1. All the bacterial isolates were maintained and grown on the respective medium (Table 1). All the bacterial isolates were screened for PHA accumulation on E2-mineral medium containing 2% w/v glucose and visualized with Nile blue A staining method [19]. The bacterial isolates that did not grow on E2-mineral medium were checked for PHA accumulation on nutrient agar plates.

2.2. Quantitative Analysis of PHA. The isolates which showed orange fluorescence on Nile blue A staining method were selected for PHA extraction. These isolates were grown in 250 mL Erlenmeyer flask containing 100 mL E2 mineral medium supplemented with glucose ($20\,g\,L^{-1}$) as the sole carbon source. The flask was incubated on an Orbitek environmental shaker (170 rpm) for 48 h at 30°C. The bacterial isolates which could not grow in E2 broth were grown in nutrient broth. The bacterial cells were harvested, and PHA was extracted using the sodium-hypochlorite method [19]. Biomass and PHA obtained were quantified gravimetrically afterwards every 24 h till a constant weight was obtained.

2.3. Characterization of PHA Using Fourier Transform Infrared Spectroscopy (FTIR). PHA samples were dissolved in chloroform and made to thin film. The FTIR spectrum of the film of polymer was recorded at 400–4000 cm^{-1} in FTIR [40].

2.4. Physiological Characterization. All the strains of *Bacillus* used in this study were tested for several physiological characteristics using the methods described in Bergey's Manual of Systematic Bacteriology [41, 42]. Tests used in the present study are listed in Table 2.

2.5. Designing Specific Primers. Complete sequences of *phaC* genes AB525783 (*B. megaterium*), CP001598 (*B. anthracis*), HM122246 (*B. cereus*), GU190695 (*B. mycoides*), CP001903 (*B. thuringiensis*), CP000903 (*B. weihenstephanensis*) and AB077026 (*Bacillus* sp. INT005) were obtained from the NCBI nucleotide database and used for designing specific primers. The sequences were aligned using Clustal-X in order to search for internal region of *phaC* gene specific for *B. megaterium* (Figure 1). A pair of oligonucleotides, forward and reverse, was selected. The primers were BmphaC015F (CGT-GCAAGAGTGGGAAAAAT) as forward and BmphaC931R (TCGCAATATGATCACGGCTA) as reverse with t_m value 63.9°C. These were synthesized by Bangalore Genei Pvt. Ltd., Bangalore, India. The sequences and positions of oligonucleotides are presented in Figure 1.

2.6. DNA Extraction. Bacterial isolates were grown in respective media broth at 30°C. DNA was extracted from 24 h old culture using the protocol described in Sambrook et al. [43].

2.7. PCR Optimization of phaC. PCR using the internal primers (Figure 1) for *phaC* was optimized as follows: PCR reactions were performed in a total volume of 50 μL. The reaction mixture contains 2 μL of genomic DNA as a template, 10X PCR buffer [100 mM Tris-HCl (pH 9), 500 mM KCl and 0.1% gelatin], 1.5 mM $MgCl_2$, 10 mM dNTP Mix, 1 μM–10 μM of each primer depending on the requirement, and 5 units/μL of *Taq* polymerase (Bangalore Genei, India). Genomic DNA of six bacterial species were used; these were *Bacillus megaterium*, *Bacillus flexus*, *Bacillus endophyticus*, *Bacillus* sp., *Paracoccus yeii*, and *Pseudomonas aeruginosa*. Reactions were carried out in a thermocycler (BIOER XP Cycler, China) consisting of initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 51 to 64°C (as required) for 1 min, elongation at 72°C for 1 min followed by 72°C for 5 min and final storage at 4°C. The specificity was optimized by adjusting annealing temperature from 51 to 64°C and primer concentration from 1 to 10 μM.

Table 1: Bacterial cultures: their source and growth medium.

Serial no.	Bacterial species	Medium	Source	16S rRNA sequence accession no.	PHA accumulation (% w/w)
1	*Aneurinibacillus migulanus* 81A1^T	NA	NRS 1137T*	ND	–
2	*B. amyloliquefaciens* 10A1	NA	BGSC 10A1*	ND	+ (18.246)
3	*B. aquimaris*	NA	MTCC6722	AF483625$	–
4	*B. coagulans* 61A1^T	NA	ATCC 7050*	DQ297928$	+ (15.020)
5	*B. cereus* 6A5	NA	ATCC 14579*	AE016877$	–
6	*B. circulans* 16A1^T	NA	ATCC 4513*	FJ560956$	+ (18.000)
7	*B. endophyticus* TMR1.22	TYGA	Coastal sand-dune (R)	HQ897169#	+ (39.409)
8	*B. firmus* 29A1^T	NA	NRS 613T*	ND	+ (20.078)
9	*B. flexus* NAMR4.1	NA	Coastal sand-dune (R)	HM026605#	+ (47.476)
10	*B. licheniformis* 5A1	NA	ATCC 8480*	ND	–
11	*B. megaterium*	NA	MTCC428	ND	+ (32.630)
12	*B. megaterium* 7A16	NA	QM B1551*	CP001983$	+ (32.814)
13	*B. megaterium* NQ-11/A2	NA	Arabian sea-continental shelf sediment sample-NCIM5334	FJ392860#	+ (61.000)
14	*B. megaterium* COL1/A6	NA	Humus sample	EU702754#	+ (65.510)
15	*B. megaterium* BLQ-2/A7	NA	sediment sample	EU924811#	+ (59.870)
16	*B. megaterium* TMR1.3.2	TYGA	Coastal sand-dune (R)	GU984576#	+ (39.356)
17	*B. megaterium* TMR1.4	TYGA	Coastal sand-dune (R)	GU951918#	+ (40.801)
18	*B. megaterium* NAMNR3.7	NA	Coastal sand-dune (NR)	GU951917#	+ (41.776)
19	*B. mojavensis*	NA	MTCC8604	AF440779$	+ (12.345)
20	*B. mycoides* 6A19	NA	ATCC 31101*	EF210306$	–
21	*B. niacin*	NA	MTCC8323	ND	+ (11.702)
22	*B. pumilus* 8A3	NA	ATCC 7061*	EU138517$	+ (23.428)
23	*B. simplex*	NA	MTCC7284	ND	+ (26.972)
24	*B. spizizenii*	NA	ATCC 6633	AB018486$	–
25	*B. subtilis* subsp. *subtilis* 3A1	NA	NCIB 3610*	ND	+ (18.729)
26	*Bacillus* sp. TMR1.10.1	TYGA	Sand-dune (R)	HM035484#	+ (51.860)
27	*Bacillus* sp. NAMNR4.4	NA	Coastal sand-dune (NR)	JX194167#	–
28	*Bacillus* sp. NAMNR3.5	NA	Coastal sand-dune (NR)	JX194166#	+ (23.624)
29	*Bacillus* sp. TMNR4.1.1	TYGA	Coastal sand-dune (NR)	JX194168#	+ (27.369)
30	*Bacillus* sp. MS4.SE3	TYGA	Sediment sample	ND	–
31	*B. thurigiensis* 164 H-14	NA	RCPFBS	ND	+ (37.812)
32	*B. weihenstephanensis* 6A24	NA	BGSC 6A24*	ND	–
33	*Geobacillus stearothermophilus* 9A20	NA	ATCC 12980*	AY608928$	+ (13.394)
34	*Lysinibacillus fusiformis* 19A1T	NA	ATCC 7055T*	AF169537$	+ (21.794)
35	*Lysinibacillus sphaericus* 13A10	NA	ATCC 12123*	ND	+ (26.785)

Table 1: Continued.

Serial No.	Bacterial species	Medium	Source	16S rRNA sequence accession no.	PHA accumulation (% w/w)
36	*Lysinibacillus* sp. KSD-4	NA	Stagnant water-MTCC3672	FJ473365[#]	+ (25.233)
37	*Marinibacillus marinus* 21A1T	NA	DSMZ 1297[*]	AJ237708[$]	+ (21.198)
38	*Paenibacillus dendritiformis* 30A2	NA	C168[*]	AB045092[$]	+ (18.859)
39	*Paracoccus yeii* TMR3.1	TYGA	Coastal sand-dune (R)	GU906275[#]	+ (28.205)
40	*Pseudomonas aeruginosa*	NA	ATCC 9027	ND	–
41	*Pseudomonas aeruginosa* TMR2.13	TYGA	Coastal sand-dune (R)	HM030825[#]	–

NA: nutrient agar; TYGA: tryptone yeast extract glucose agar; ATCC: American Type Culture Collection, USA; MTCC: Microbial Type Culture Collection, India; NCIM: National Collection of Industrial Micro-organisms, India; RCPFBS: Russian commercial powder formulations Bacticide and Sphericide; BGSC: Bacillus Genetic Stock Center, Columbus; NRRL: Northern Regional Research Laboratory, USA; NCIB: National Collection of Industrial, Marine and Food Bacteria, Scotland; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; NRS: Northern Research Station, USA; [*] cultures obtained from BGSC; [#] sequences obtained in current study; [$] sequences obtained from NCBI; R: Rhizosphere; NR: nonrhizosphere; PHA: polyhydroxyalkanoate; ND: not done; + and −: Nile Blue A staining method; +: accumulating PHA; −: negative for PHA accumulation; PHA accumulation (% w/w), gravimetric method.

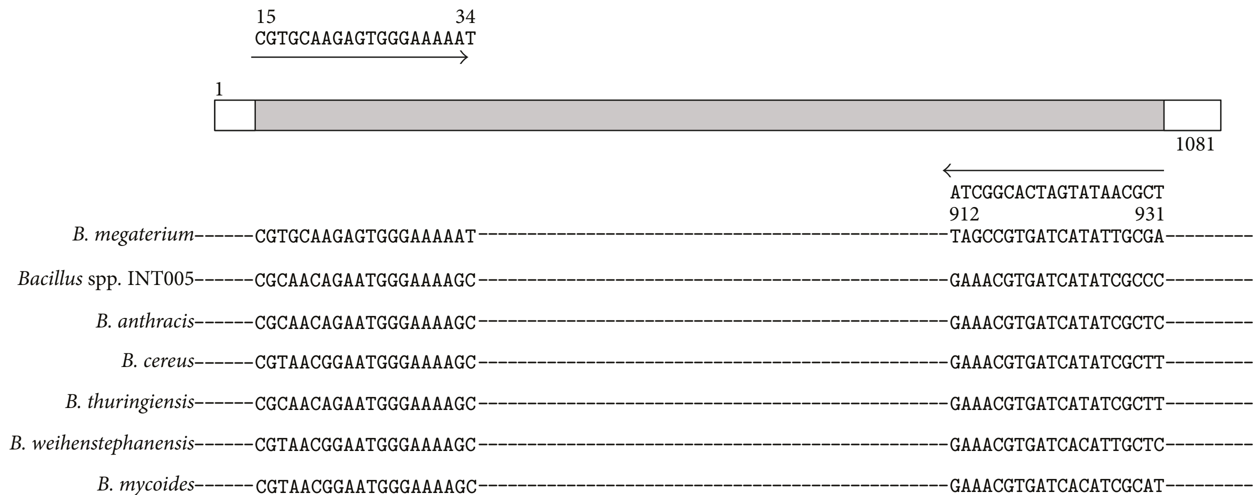

Figure 1: Location of forward and reverse primers on *phaC* gene of *B. megaterium*. The numbers indicate the positions of the primers on the *phaC* gene. The arrow indicates the direction of forward and reverse primers on the gene. Accession numbers of the sequences used to draw the diagram are given in the text.

2.8. Detecting PCR Products. Electrophoresis on 1% (w/v) agarose gel was used for detecting PCR amplification products [43]. 100 or 500 bp ladders were used as DNA size marker. Run conditions were 100 volts for 2 hours. The gel was stained with ethidium bromide solution (0.5 μg/mL). Amplified DNA fragments were visualized under UV light and recorded using a Gel-Doc Alpha Imager (Alpha Innotech, USA).

2.9. Validating PCR. Amplified products from strains *B. megaterium* TMR1.3.2 and *B. megaterium* TMR1.4 under optimum conditions having expected size were purified using purification kit (Quaigen, India) and were sequenced using forward primer (BmphaC015F) at Bangalore Genei, India. Sequences obtained were compared with sequences in the NCBI nucleotide database using Blastn. Sequences were deposited in GenBank. Amplified nucleotide sequences were analyzed and aligned with the reference sequences of *phaC* of various bacterial species using ClustalX [44], and neighbor-joining (NJ) tree was obtained with 1000 seeds and 10000 bootstraps. The final tree obtained was rooted and drawn using MEGA 4.0 [45].

Optimized PCR conditions with 1 μM primer concentration and 64°C annealing temperature were used for the amplification of 0.9 kb from *phaC* of *B. megaterium* by SYBR green based real-time PCR [46]. The melting curve of the amplicon was performed from 55 to 94°C for detecting t_m of amplicon.

All the bacterial isolates mentioned in Table 1 were used for PCR amplification of *phaC* at optimum conditions (Method I) of 1 μM primer concentration and 64°C annealing temperature and suboptimal conditions (Method II) of 10 μM

Table 2: Physiological characteristics of bacterial cultures belonging to Bacillales.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Pigmented colony	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	+	+	−	−
Gram character	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Swell sporangia	−	−	−	−	−	−	+	+	+	+	−	+	+	−	−	+	+	+	−	+	+	−	+	+	−	−	−	+	−	−	−	−	−	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
Oxidase	−	−	−	+	+	−	−	+	+	+	+	+	+	+	−	−	−	+	+	−	+	−	−	−	+	+	−	+	+	−	+	+	−	−
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	−	+	+	−	+	−	−	−	−	−	+	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+	−	−	+	−	−	+	+
VP	−	−	−	−	−	+	−	−	−	−	nd	+	nd	+	+	−	−	−	+	−	+	+	−	−	+	+	+	+	+	−	+	+	+	−
Nitrate reduction	−	+	−	−	−	+	−	−	−	−	−	−	+	−	+	−	−	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+
Acid from																																		
D-glucose	+	+	+	+	+	+	+	−	−	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	−	+	+	+	−	−	−	−	−	+	−	−	−	−	+	−	+	+	−	+	−	+	−	+	−	−	+	+	−	−	−	−	−
Glycerol	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	+	+	+	+	−	−	+	+	+	+	−	+
Trehalose	+	+	+	+	+	+	nd	−	−	−	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
D-xylose	+	+	+	+	−	−	−	−	−	−	+	−	−	+	+	+	−	+	−	−	+	−	+	+	+	+	−	+	+	−	+	+	−	−
Hydrolysis of																																		
Casein	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+
Gelatin	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	−	+	+	+	+	−	−	−	−	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Aesculin	+	+	+	+	−	+	+	−	−	−	+	nd	−	nd	nd	−	−	nd	+	−	+	nd	+	+	+	nd	+	nd	+	+	nd	nd	+	nd
Urea	−	+	+	+	+	+	−	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	+	−	+	−	+	−	−	−	−	−	−	−
Citrate utilization	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+	+	−	+	+	+	−
Growth in NaCl																																		
2%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+
7%	+	+	+	+	+	+	−	+	−	+	+	−	−	+	+	+	−	+	+	+	−	+	+	+	+	+	+	−	+	−	+	+	+	+
10%	−	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	−	+	+	+	−	−	+	+	−	+	−	−	+	−	−	−	+	+
PHA accumulation	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	−	+	−	+	−	+	+	−	+	−	+	+	+	−	+	+	−
Growth at temp.																																		
10°C	+	−	−	−	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	+	+	−	+	+	−	−
20°C	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50°C	+	+	−	+	+	−	+	−	+	−	−	−	+	+	+	−	−	+	+	−	+	+	+	+	+	+	−	−	+	−	+	+	−	+
60°C	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

All the organisms were positive for endospore formation, catalase, and growth at 30°C; +: positive; −: negative; nd: not determined; 1: *B. megaterium* MTCC428 and *B. megaterium* QMB1551; 2: *B. megaterium* TMR1.3.2; 3: *B. megaterium* TMR1.4; 4: *B. megaterium* NAMNR3.7, *B. megaterium* NQ-11/A2, *B. megaterium* Col1/A6 and *B. megaterium* BLQ-2/A7; 5: *B. flexus* NAMR4.1; 6: *B. weihenstephanensis*; 7: *Geobacillus stearothermophilus*; 8: *Lysinibacillus fusiformis*; 9: *Lysinibacillus sphaericus*; 10: *Lysinibacillus* sp. KSD-4; 11: *Marinibacillus marinus*, 12: *Paenibacillus dendritiformis*; 13: *Aneurinibacillus migulanus*; 14: *Bacillus* sp. NAMNR3.5; 15: *Bacillus* sp. TMNR4.1.1; 16: *B. endophyticus* TMR1.22; 17: *Bacillus* sp. TMR1.10.1; 18: *Bacillus* sp. NAMNR4.4; 19: *B. amyloliquefaciens*; 20: *B. aquimaris*; 21: *B. coagulans*; 22: *B. cereus*; 23: *B. circulans*; 24: *B. firmus*; 25: *B. licheniformis*; 26: *B. mojavensis*; 27: *B. mycoides*; 28: *B. niacini*; 29: *B. pumilus*; 30: *B. simplex*; 31: *B. spizizenni*; 32: *B. subtilis*; 33: *B. thuringiensis*; 34: *Bacillus* sp. MS4.SE3.

Table 3: Optimization of PCR condition for amplification of internal region of *phaC* gene using primer set described in Figure 1.

Primer (μM)	Annealing temperature (°C)	Bacterial species											
		B. megaterium (MTCC428) PHA +		*B. flexus* (NAMR4.1) PHA +		*B. endophyticus* (TMR1.22) PHA +		*Bacillus* sp. (NAMNR4.4) PHA −		*P. yeii* (TMR3.1) PHA +		*P. aeruginosa* (TMR2.13) PHA −	
		0.9 Kb	MB	0.9 Kb	MB	0.9 Kb	MB	0.9 Kb	MB	0.9 Kb	MB	0.9 Kb	MB
1	51	+	−	−	−	−	−	−	−	−	−	−	−
	55	+	−	−	−	−	−	−	−	−	−	−	−
	60	+	−	−	−	−	−	−	−	−	−	−	−
	64	+	−	−	−	−	−	−	−	−	−	−	−
2.5	51	+	−	−	−	−	−	−	−	−	−	−	−
	55	+	−	−	−	−	−	−	−	−	−	−	−
	60	+	−	−	−	−	−	−	−	−	−	−	−
	64	+	−	−	−	−	−	−	−	−	−	−	−
5	51	+	*	−	+	−	+	−	−	−	−	−	−
	55	+	*	−	+	−	+	−	−	−	−	−	−
	60	+	*	−	+	−	+	−	−	−	−	−	−
	64	+	*	−	−	−	−	−	−	−	−	−	−
10	51	+	*	−	+	−	+	−	−	−	−	−	−
	55	+	*	−	+	−	+	−	−	−	−	−	−
	60	+	*	−	+	−	+	−	−	−	−	−	−
	64	+	*	−	−	−	−	−	−	−	−	−	−

PHA +: polyhydroxyalkanoate accumulating bacteria; PHA −: bacteria not accumulating polyhydroxyalkanoate; ∗: double band; MB: multiple band; +: positive; −: absent.

and 51°C. Presence of genomic DNA was confirmed using amplification of 16S rRNA gene using universal primers as a positive control [12]. Entire experimental sets were repeated thrice to determine the reproducibility of Method I and Method II.

A blind folded test was carried out using a few bacterial isolates. *B. megaterium* BLQ-2/A7 and *B. simplex* MTCC7284, known for PHA accumulation, were taken as positive controls. Other six bacteria were randomly selected from genus *Bacillus*. These were not reported for PHA accumulation earlier. Three of these strains were *B. aquimaris* MTCC6722, *B. mojavensis* MTCC8604, and *B. niacin* MTCC8323 and three were unidentified *Bacillus* sp. NAMNR3.5, *Bacillus* sp. TMNR4.1.1, and *Bacillus* sp. MS4.SE3 (Table 1). These bacterial isolates were tested for biochemical characteristics, PHA accumulation, and PCR amplification using Method I and Method II. All the experiments were repeated three times.

3. Results

3.1. PHA Accumulation. All the bacterial cultures used in the present investigation were tested for their ability to accumulate PHA on solidified E2-mineral medium with 2% w/v glucose (Table 1). *B. aquimaris* and *B. niacin* did not show any growth on E2-mineral medium. These strains were grown on nutrient agar and screened for PHA accumulation. All the strains of *B. megaterium* showed PHA accumulation. *Aneurinibacillus migulanus*, *B. aquimaris*, *B. cereus*, *B. licheniformis*, *B. mycoides*, *B. spizizenii*, *Bacillus* sp. NAMNR4.4, *Bacillus* sp. MS4.SE3, *B. weihenstephanensis*, and *Pseudomonas aeruginosa* did not show PHA accumulation. The remaining strains showed PHA accumulation.

3.2. Quantitative Analysis and Characterization of PHA. The PHA content of all the bacterial isolates was found between 10 to 66% w/w (Table 1). PHA content varied among the isolates of *B. megaterium*. FTIR spectroscopy of PHA extracted from all the bacterial isolates showed intense absorptions typical to PHA at 1724–1740 cm^{-1} and at 1280 cm^{-1} corresponding to C=O and C–O stretching groups in ester, respectively.

3.3. Physiological Characteristics. The physiological characteristics of the *Bacillus* strains are listed in Table 2. All the *Bacillus* strains are endospore producers, catalase positive and showed growth at 30°C. Different strains of *B. megaterium* including the type strain QM B1551 showed variations in the physiological tests and accordingly are separated in Sections 1 to 4 in Table 2.

3.4. PCR Optimization. PCR optimization of internal region of *phaC* BmphaC015F and BmphaC931R primers were used for amplifying *phaC* gene. Different annealing temperatures such as 51, 55, 60, and 64°C and different primer concentrations such as 1, 2.5, 5, and 10 μM were used for optimization (Table 3). It was seen that, in *B. megaterium* at different annealing temperatures with primer concentrations of 1 and

2.5 μM, there was amplification of a single band of 0.9 kb. With primer concentrations of 5 and 10 μM along with 0.9 kb an additional faint band of 1.9 kb was present. In *B. flexus* and *B. endophyticus* at annealing temperatures 51, 55, 60, and 64°C with primer concentrations 1 and 2.5 μM there was no amplification, and, with primer concentrations of 5 and 10 μM, multiple bands were seen in all the temperatures except at 64°C. PHA accumulating *P. yeii* and PHA negative *P. aeruginosa* and *Bacillus* species gave no amplification with any combination tested. It was observed that the 0.9 kb fragment was amplified only in *B. megaterium* and not amplified in any other bacterial isolate used in this study. When the primer concentration was increased above 5 μM with annealing temperature ≤60°C, multiple bands were detected in PHA accumulating *Bacillus* species, but no amplification was detected in Gram-negative PHA accumulating *P. yeii*, PHA negative *Bacillus* species, and *P. aeruginosa*. The optimum conditions chosen for PCR amplification of *phaC* of *B. megaterium* were annealing temperature = 64°C and primer concentration = 1 μM.

3.5. Validation of Method. Observations of PCR amplifications using primer set performed with all the bacterial cultures at optimal conditions (Method I) are seen in Figures 2 and 4(a) and under suboptimal conditions (Method II) in Figures 3 and 4(b). PCR amplification of *phaC* in all the strains of *B. megaterium* was of a single band of 0.9 kb fragment in Method I (1 μM primer concentration and 64°C annealing temperature). No amplification was detected in bacterial species other than *B. megaterium*. 16S rRNA gene was amplified in all the bacterial isolates.

PCR amplification in Method II (10 μM primer concentration and 51°C annealing temperature) showed presence of two bands in all the strains of *B. megaterium* with sizes 0.9 kb and 1.9 kb. Interestingly, members of Bacillales accumulating PHA showed multiple bands of nonspecific amplicons unique to their respective species. Strains of *B. licheniformis*, *B. cereus*, *B. mycoides*, and *B. weihenstephanensis* did not show any amplification in Method II. Other than *B. megaterium*, none of the isolates showed presence of the 0.9 kb band of amplification. All other bacterial species tested in present study did not show any band in PCR amplification. The banding pattern showed complete reproducibility during repeated extractions and amplifications using Method I and Method II. A two-percent deviation was noted in molecular weights of bands.

Results of PCR amplification of Method I and Method II using eight additional bacterial isolates as a part of the blind folded experiment are seen in Figure 5. In Method I only the strain of *B. megaterium* BLQ-2/A7 showed amplification of 0.9 kb fragment, whereas no amplification was observed with other bacterial strains. Method II showed amplification of two bands (0.9 kb and 1.9 kb) in *B. megaterium*, and nonspecific bands were observed in PHA accumulating *B. mojavensis* MTCC8604, *B. niacin* MTCC8323, *B. simplex* MTCC7284, *Bacillus* sp. NAMNR3.5, and *Bacillus* sp. TMNR4.1.1. The strains *B. aquimaris* MTCC6722 and *Bacillus* sp. MS4.SE3 showed no PHA accumulation and did not give amplification with Method II.

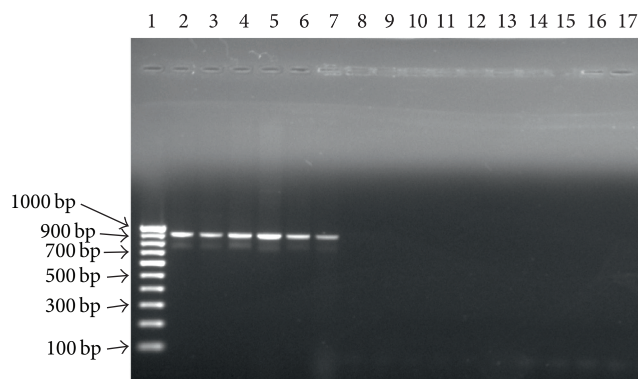

Figure 2: PCR amplification of 0.9 kb internal region of *phaC* gene under optimum condition (Method I), that is, 1 μM of each primer concentration and 64°C annealing temperature, where Lane 1—molecular weight marker; 2—*Bacillus megaterium* MTCC428; 3—*Bacillus megaterium* TMR1.3.2; 4—*Bacillus megaterium* TMR1.4; 5—*Bacillus megaterium* NAMNR3.7; 6—*Bacillus megaterium* NQ-11/A2; 7—*Bacillus megaterium* COL1/A6; 8—*Bacillus flexus* NAMR4.1; 9—*Bacillus endophyticus* TMR1.22; 10—*Bacillus thuringiensis* 164(H-14); 11—*Bacillus sphaericus* KSD-4; 12—*Bacillus* sp. TMR1.10.1; 13—*Paracoccus yeii* TMR3.1; 14—*Bacillus spizizenii* ATCC 6633; 15—*Pseudomonas aeruginosa* ATCC 9027; 16—*Bacillus* sp. NAMNR4.4; 17—*Pseudomonas* aeruginosa TMR2.13.

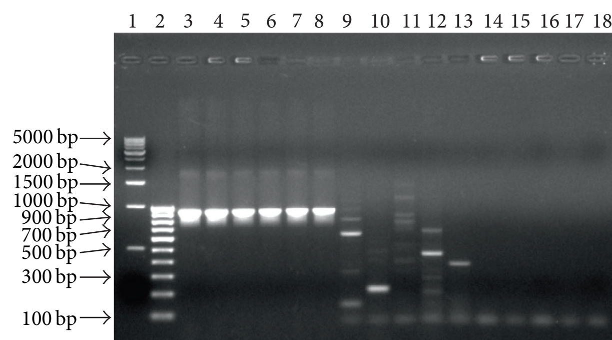

Figure 3: PCR amplification of 0.9 kb internal region of *phaC* gene in Method II, that is, 10 μM of each primer concentration and 51°C annealing temperature, where Lane 1—molecular weight marker (500 bp); 2—molecular weight marker (100 bp); 3—*Bacillus megaterium* MTCC 428; 4—*Bacillus megaterium* TMR1.3.2; 5—*Bacillus megaterium* TMR1.4; 6—*Bacillus megaterium* NAMNR3.7; 7—*Bacillus megaterium* NQ-11/A2; 8—*Bacillus megaterium* COL1/A6; 9—*Bacillus flexus* NAMR4.1; 10—*Bacillus endophyticus* TMR1.22; 11—*Bacillus thuringiensis* 164(H-14); 12—*Bacillus sphaericus* KSD-4; 13—*Bacillus* sp. TMR1.10.1; 14—*Paracoccus yeii* TMR3.1; 15—*Bacillus spizizenii* ATCC 6633; 16—*Pseudomonas aeruginosa* ATCC 9027; 17—*Bacillus* sp. NAMNR4.4; 18–*Pseudomonas aeruginosa* TMR2.13.

The dendogram constructed using multiple banding patterns of amplicon showed all the strains of Bacillales clustered differently (Figure 6). Each pattern of band showed a very little similarity to each other. Further, Method II gave multiple banding patterns for PHA accumulating *Bacillus subtilis* subsp. *subtilis* but did not give any amplification for non-PHA accumulator *Bacillus subtilis* subsp. *spizizenii*.

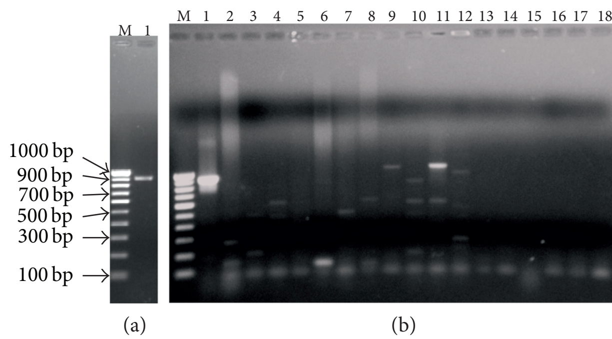

Figure 4: (a) PCR amplification of 0.9 kb internal region of *phaC* gene under optimum condition (Method I), where Lane M—molecular weight marker (100 bp); 1—*Bacillus megaterium* QM B1551. (b) PCR amplification of 0.9 kb internal region of *phaC* gene in Method II. Where Lane M—molecular weight marker (100 bp); 1—*Bacillus megaterium* QM B1551; 2—*Bacillus pumilus* ATCC 7061; 3—*Bacillus amyloliquefaciens* BGSC 10A1; 4—*Bacillus Coagulans* ATCC 7050; 5—*Bacillus firmus* NRS 613T; 6—*Geobacillus stearothermophilus* ATCC 12980; 7—*Bacillus subtilis* subsp. *subtilis* NCIB 3610; 8—*Paenibacillus dendritiformis* C168; 9—*Marinibacillus marinus* DSMZ 1297; 10—*Lysinibacillus sphaericus* ATCC 12123; 11—*Lysinibacillus fusiformis* ATCC 7055T; 12—*Bacillus circulans* ATCC 4513; 13—*Bacillus mycoides* ATCC 31101; 14—*Bacillus weihenstephanensis* BGSC 6A24; 15—*Bacillus licheniformis* ATCC 8480; 16—*Aneurinibacillus migulanus* NRS 1137T; 17–*Bacillus cereus* ATCC 14579; 18—negative control.

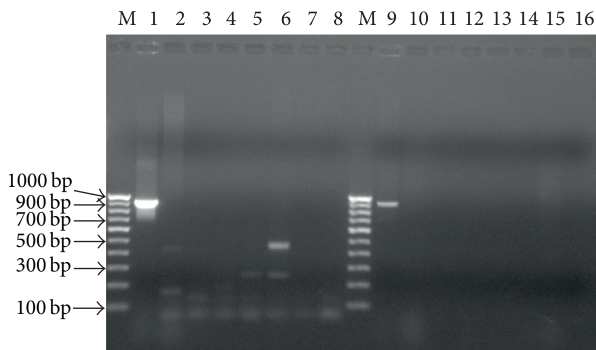

Figure 5: PCR amplification of 0.9 kb internal region of *phaC* in Method II (Lane 1–8) and Method I (Lane 9–16), where Lane M—molecular weight marker (100 bp); 1—*Bacillus megaterium* BLQ-2/A7; 2—*Bacillus niacini* MTCC 8323; 3—*Bacillus simplex* MTCC 7284; 4—*Bacillus mojavensis* MTCC 8604; 5—*Bacillus* sp. NAMNR3.5; 6—*Bacillus* sp. TMNR4.1.1; 7—*Bacillus aquimaris* MTCC 6722; 8—*Bacillus* sp. MS4.SE3; M–molecular weight marker (100 bp); 9—*Bacillus megaterium* BLQ-2/A7; 10—*Bacillus niacini* MTCC 8323; 11—*Bacillus simplex* MTCC 7284; 12—*Bacillus mojavensis* MTCC 8604; 13—*Bacillus* sp. NAMNR3.5; 14—*Bacillus* sp. TMNR4.1.1; 15—*Bacillus aquimaris* MTCC 6722; 16—*Bacillus* sp. MS4.SE3.

The clusters formed due to multiple bands among members of Bacillales were different from the clustering obtained with 16S rRNA gene of these species. Method II therefore was able to discriminate PHA accumulating Bacillales up to species level. Although the results were reproducible, it is recommended that it should be used cautiously in combination with other methods.

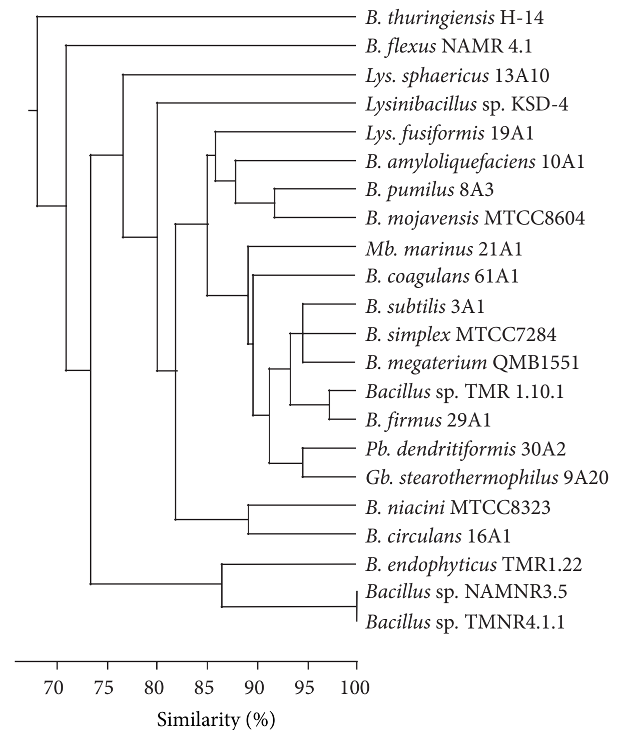

Figure 6: Dendogram of multiple banding patterns obtained in different PHA accumulating *Bacillus* species. Clustering was achieved by unweighted pair group average linkage (UPGMA). The computations were performed using the Probiosys Software.

The melting curve of amplicon from *B. megaterium* TMR1.4 showed the presence of a single peak at 85°C. This confirms the specificity of the primers for the amplification of 0.9 kb of *phaC* in *B. megaterium*.

PCR products of 0.9 kb obtained with *Bacillus megaterium* TMR1.3.2 and *Bacillus megaterium* TMR1.4 were sequenced and deposited in GenBank with accession numbers JF423932 and JF423933, respectively. Nucleotide blast of these sequences showed 100% similarity with *phaC* gene of *B. megaterium*. Pairwise alignment of sequences obtained from *B. megaterium* TMR1.4 with *phaC* of other *Bacillus* sp. showed 73–75% similarity with *B. anthracis*, *B. cereus*, *B. mycoides*, *B. thuringiensis*, and *B. weihenstephanensis*. Phylogenetic tree constructed using these sequences with sequences of *phaC* of various species showed alignment of the sequences with *phaC* of *B. megaterium* (Figure 7).

4. Discussion

Polyhydroxyalkanoate production in bacteria is mediated by polyhydroxyalkanoate synthase. This enzyme has been classified into four classes, that is, Class I to Class IV. In *B. megaterium*, rapid PHA production occurs after a long lag phase. A 4,104 bp cluster consists of five *pha* genes responsible for PHA synthesis in this bacterium. It consists of *phaP*, *phaQ*, *phaR*, *phaB*, and *phaC*. PHA synthase of *B. megaterium* is a heterodimer and belongs to Class IV. This PHA synthase consists of 40 kDa PhaC and 20 kDa PhaR. Genes of both these proteins are parts of an operon consisting

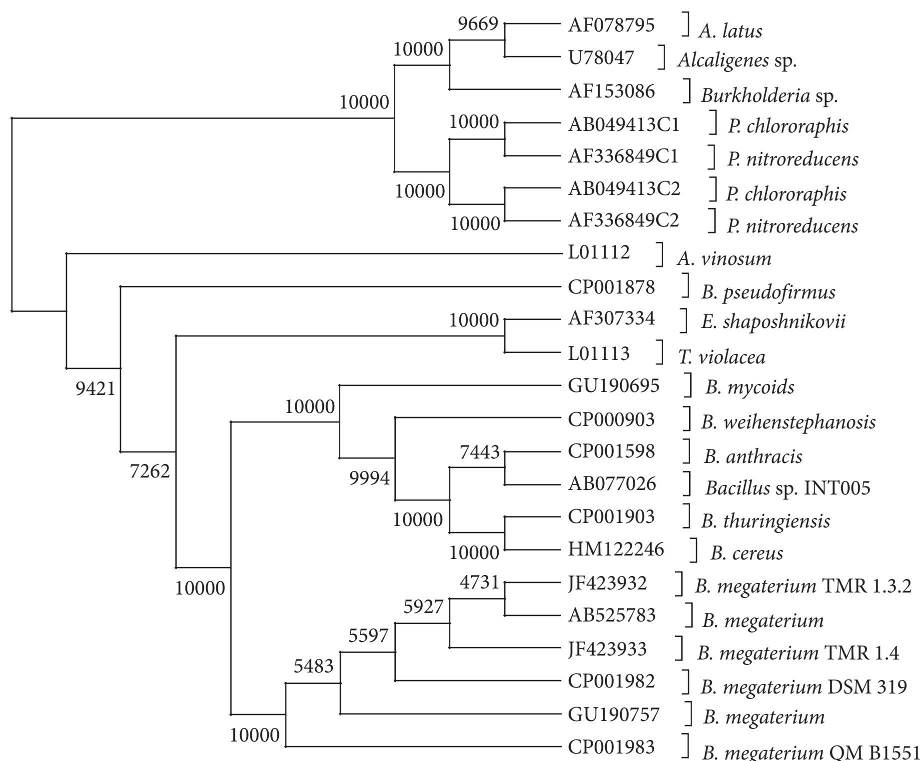

Figure 7: Phylogenetic tree of *phaC* genes of various bacterial species. The tree was constructed using 1000 seeds and 10000 bootstraps. Except for the strains *B. megaterium* TMR1.3.2 and *B. megaterium* TMR1.4 all the sequences used were complete. The node presents the value of bootstrap obtained out of 10000.

of *phaR*, *phaB*, and *phaC*. These three genes are transcribed as tricistronic mRNA. The *phaB* encodes for acetoacetyl CoA reductase, which provides hydroxybutyryl CoA for PHA synthase [2, 47, 48]. PhaQ is a DNA binding protein, which represses its own synthesis. Once PHB is produced, it binds to PhaQ resulting in the removal of repression on transcription of *phaQ* and *phaP* [49]. The *phaQ* and *phaP* are transcribed independently as a single mRNA 1.2 kb that is presumably processed to give 0.7 kb transcript responsible synthesis of phasins protein—PhaP. Once the phasins are produced, the granule formation of PHA in *B. megaterium* gets stabilized [47]. *Bacillus megaterium* contains budding granules of PHB in which PHA synthase is covalently bound [12].

Nucleotide sequences of *phaC* gene from *B. megaterium* showed 73–75% homology with *phaC* gene of other polyhydroxyalkanoate accumulating *Bacillus* sp. This indicates the uniqueness of the sequences of *phaC* of *B. megaterium*. The differences in the gene sequences serving as recognition of unique regions present at 15–34 and 912–931 on *phaC* were utilised to design the primers for identification of *B. megaterium*. Earlier Shamala et al. [50] designed a set of primer using *phaC* gene sequences of *B. megaterium* for the detection of PHA producing *Bacillus* sp., where the amplification product was 590 bp in different PHA accumulating *Bacillus* sp. including *B. megaterium*. In the present study the internal primers designed are unique and bind in the *phaC* gene at 15–34 (forward primer) and 912–931 (reverse primer). PCR amplification using this primer set results in single amplicon of 0.9 kb seen only in *B. megaterium* at specified optimum conditions. Specificity of the primers was validated by sequencing of 0.9 kb PCR amplification product from *B. megaterium,* that gave 100% similarity with *phaC* of *B. megaterium*, and real-time PCR that gave T_m of the amplicon as 85°C. Recently genomes of two strains of *Bacillus megaterium* namely QM B1551 and DSM 319 were sequenced completely [16]. One strain, namely, QM B1551 was incorporated for cross verification of specificity of the primers and method thereof. The method showed amplification of 0.9 kb fragment with this strain. This confirms the specificity of the primers. The ubiquity of *B. megaterium* in the natural environment and its emerging industrial importance could help if the identification of new potential strains of this bacterium becomes rapid and easier. In comparison to routinely used molecular methods for identification of *B. megaterium*, the present method is rapid and specific. Further, this method does not require sequencing of amplicon or any additional test to confirm its identification.

PCR based randomly amplified polymorphic DNA (RAPD) technique has been used for molecular typing and identification among closely related species of the genus *Bacillus* [30, 31]. The pattern of amplified DNA fragments produce during RAPD-PCR provides information on genetic variability between organisms of different species. Interestingly, in Method II *B. megaterium* and other PHA producing Bacillales gave multiple banding patterns of nonspecific amplicon unique to respective species. Members of Bacillales such as *Lysinibacillus*, *Marinibacillus*, *Geobacillus*, *Aneurinibacillus*, *Paenibacillus*, and all the species of *Bacillus* reported

for accumulation of PHA were included. Although, *B. licheniformis*, *B. cereus*, *B. mycoides*, and *B. weihenstephanensis* were reported as PHA accumulators, the isolates of these species used in this study neither showed PHA accumulation nor any amplification in Method II.

Different methods have been used for the identification of *B. megaterium* which are both laborious and time consuming. During the last decade a number of new species of the genus *Bacillus* have been described showing a very close similarity to *B. megaterium*. This has resulted in a difficulty in accurate nomenclature of the isolates. Even though several methods are used, the identification is limited to genus level only [19, 27]. It is known that, among the members of Bacillales, *B. flexus* and *B. simplex* show very close similarity with *B. megaterium* in physiological characteristics and 16S rRNA sequencing, but in this study PCR amplification of the 0.9 kb region of *phaC* gene under optimum condition resulted in no amplification in *B. flexus* and *B. simplex*. Pairwise alignment of 16S rRNA sequences of *B. megaterium* and *B. flexus* showed 11 nucleotide differences in the hypervariable region in between 150 and 200 bp region of their 16S rRNA gene. Inconsistency in biochemical test results and errors in sequencing of 16S rRNA gene may lead to wrong identification. Hence, this method can be ideally used to differentiate *B. megaterium* from *B. flexus* and *B. simplex*. With the increasing importance of *B. megaterium* in the field of biotechnology, the ambiguity observed with its identification by conventional biochemical and molecular methods would limit its application. The present simple yet rapid method counteracts these problems and is thus a suitable alternative for the accurate identification of the organism.

Like *B. megaterium* other members of Bacillales are attractive industrial organisms with known capabilities to produce enzymes, recombinant proteins, antibiotics, purine nucleotides, insecticidal proteins, vitamins, sugars biopolymer, and biofertilizers [51–57]. These strains are gaining interest for economic production of these compounds for a variety of reasons including high growth rates, short fermentation cycle times, ability to tolerate wide pH and temperature ranges, easy to maintain in spore forms, capacity to secrete proteins into the extracellular medium, amenable to genetic engineering, and the GRAS (generally regarded as safe) status [55]. Further, the suboptimal conditions described as Method II were suitable for rapid differentiation for identification of polyhydroxyalkanoate accumulating members of Bacillales.

Abbreviations

PHA: Polyhydroxyalkanoate
PHB: Polyhydroxybutyrate
phaC: Polyhydroxyalkanoate synthase
PCR: Polymerase chain reaction
RT-PCR: Real-time PCR
RAPD: Randomly amplified polymorphic DNA.

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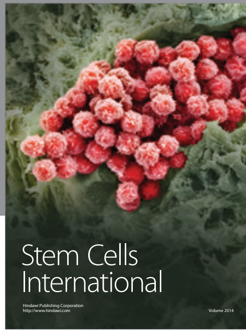

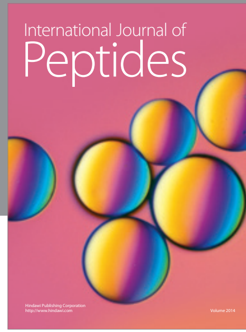

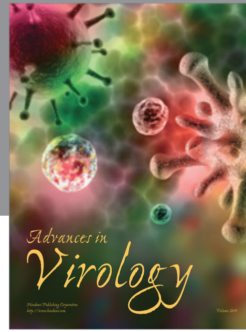

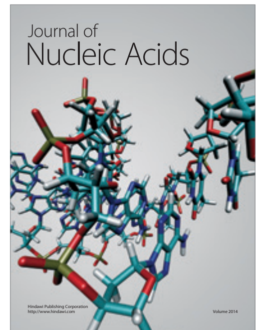

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