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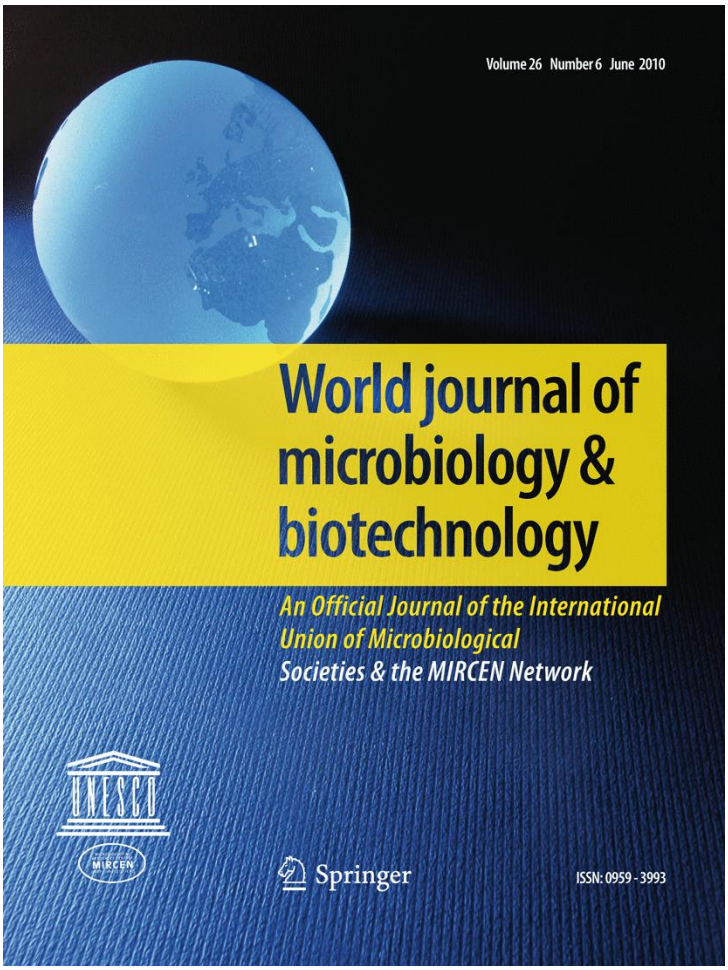

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ORIGINAL PAPER

Polyhydroxyalkanoate (PHA) accumulating bacteria from the gut of higher termite *Macrotermes carbonarius* (Blattodea: Termitidae)

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Abstract The continuous quest for bacterial strains capable of accumulating polyhydroxyalkanoate (PHA) utilizing cheaper and renewable carbon source prompted us to explore newer and diverse environments like the gut of termites. Among the bacterial strains isolated from the gut of higher termite *Macrotermes carbonarius*, three strains were found to accumulate PHA, as observed by microscopic studies and PHA production experiments. Among them, strain MC1 with rapid growth and higher PHA accumulation was selected for further studies. API kit-50 CHB and 16S rRNA gene sequence analysis results indicated the strain to have 99% homology with *Bacillus megaterium* and *Bacillus flexus*. *Bacillus* sp. MC1 was able to accumulate PHA during the growth phase utilizing different carbon sources like glucose, fructose, sodium acetate, sodium valerate and 1,4-butanediol. Gas chromatography analysis of the polymer has shown it to be basically composed of poly (3-hydroxybutyrate) (PHB). Growth associated PHB biosynthesis was best in the presence of sodium acetate with 39 wt% after 16 h of cultivation. Though previous studies provided evidence confirming the presence of PHA producing bacteria in termite gut, isolation and characterization of these strains in pure culture has not been documented yet. Presence of other morphotypes in the termite gut with PHA like granular inclusions was evident from the transmission electron microscopy studies. This is a novel report and shows the feasibility of using potent strains capable of utilizing lignocellulosic degradation products as a renewable carbon source for the production of PHA in the future.

Keywords *Bacillus* sp. · Lignocellulose · *Macrotermes carbonarius* · Polyhydroxyalkanoates (PHA) · Termite gut

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Introduction

Polyhydroxyalkanoates (PHAs) are homo or hetero polyesters of hydroxyalkanoates synthesized by a large number of bacteria in the presence of excess carbon and under conditions of nutrient stress (Anderson and Dawes 1990). These polyesters have gained increasing world wide attention due to their similar physical and material properties as conventional plastics in addition to their biocompatibility and biodegradable properties. Poly(3-hydroxybutyrate) (PHB) is the most commonly found and extensively studied member of PHA family. The ability to accumulate PHB has been shown to enhance the survivability of bacteria during prolonged period of starvation and/or under stress conditions (Ruiz et al. 2001). In the constant search for PHB producing bacteria with attractive characteristics with respect to production, material properties and substrate utilization, researchers around the world have been frequently screening newer environments for isolation of potent strains. In the process, various diverse environments such as soil, municipal sewage sludge (Reddy et al. 2009), fish gut (Kaynar and Beyatli 2009), marine environments (Arun et al. 2009), palm oil mill effluent (Alias and Tan 2005), etc. have been used as source for the isolation of PHB accumulating bacteria.

The occurrence of PHB in an environmental sample provides physiological evidence for the presence of

prokaryotic microorganisms capable of synthesizing this storage material (Findlay and White 1983). It also indicates suitable growth conditions for bacteria and the presence of sufficiently higher concentrations of organic carbon sources that can be assimilated and polymerized into PHB. Termite gut is one such ideal environment to be explored for the growth and proliferation of PHA accumulating bacteria. Termites are known to thrive well in terrestrial ecosystems and play significant role in the biorecycling of lignocellulose (Ohkuma 2003). *Macrotermes carbonarius,* a higher termite from the subfamily Macrotermitinae was considered for the present study as these termites feed on plant biomass, a diet rich in carbon and low in nitrogen. This C: N ratio provides an ideal environment for the growth of PHA accumulating bacteria. Our previous study on the gut homogenate had confirmed the presence of PHA in its gut (Sudesh et al. 2008). Moreover, their abundant presence in various locations within the University Sains Malaysia campus made it suitable for fresh specimen collection and observation.

Termites harbor dense and diverse population of microbial symbionts in their guts, which help in the efficient decomposition of lignocellulosic materials. Among the gut symbionts several groups of bacteria have been known to inhabit the gut of termite. Various physiologically distinct groups residing in the gut flora have been isolated and found to be mainly comprised of methanogens (Leadbetter et al. 1998), enteric bacteria (Adams and Boopathy 2005), sulphate-reducing bacteria (Fröhlich et al. 1999), acetogens (Leadbetter et al. 1999), nitrogen-fixers (Lilburn et al. 2001; Doolittle et al. 2008), cellulolytic bacteria (Wenzel et al. 2002) and hemicellulolytic bacteria (Schäfer et al. 1996). Recent studies have also shown bacteria to play an important role in cellulose digestion in flagellate-free termites (Tokuda and Watanabe 2007) and these microbes play important role in the survivability of the hosts.

However, little is known about the presence of PHA producing bacteria in the termite gut. Although molecular methods have revealed the presence of bacteria that are phylogenetically related to PHA producers (Thongaram et al. 2005), and gas chromatography and NMR analysis of whole termite gut homogenate provided evidence for the occurrence of PHA (Sudesh et al. 2008), there is still no concrete evidence for the occurrence of PHA producing bacteria in termite gut**.** In addition, as termites are known to feed on lignocellulosic matter, there is a good probability of occurrence of PHA synthesizing bacterial strains with the ability to assimilate lignocellulosic degradation products, the most abundant renewable carbon source on the planet. In this study, we report for the first time successful isolation and characterization of PHA producing bacteria from the gut of a higher termite *Macrotermes carbonarius*.

Methods

Bacterial growth media and culture conditions

Termite gut isolates were grown and maintained in Nutrient Broth (NB) and Nutrient Agar (NA) (HIMEDIA), respectively. In this study, 26 g l^{-1} of NB was used and the cells were grown at 30°C with constant agitation at 150 rpm. PHA analysis with one-stage cultivation method was carried out using Minimal Salts Medium (MSM). MSM consisted of the following compounds dissolved in deionized water: KH_2PO_4 (1.5 g l^{-1}), Na_2HPO_4 (3.6 g l^{-1}), NH_4Cl (0.5 g l^{-1}), 1 ml l^{-1} of $MgSO_4{\cdot}7H_2O$ (0.1 mM) and was supplemented with 1 ml l^{-1} of trace element solution. Trace element solution consisted of the following compounds dissolved in 0.1 M HCl: $FeSO_4{\cdot}7H_2O$ (2.78 g l^{-1}), $MnCl_2{\cdot}4H_2O$ (1.98 g l^{-1}), $CoSO_4{\cdot}7H_2O$ (2.81 g l^{-1}), $CaCl_2{\cdot}2H_2O$ (1.67 g l^{-1}), $CuCl_2{\cdot}2H_2O$ (0.17 g l^{-1}) and $ZnSO_4{\cdot}7H_2O$ (0.29 g l^{-1}). Two-stage cultivation method was carried out with similar MSM composition, but for the omission of NH_4Cl. For growth profile studies, Luria–Bertani medium (LB) and NB medium [13, 26 and 26 g l^{-1} supplemented with 1% (w/v) of glucose] were used.

Isolation and cultivation of bacteria from the termite gut

Gut bacteria were isolated within 1–2 h of termite collection from the site. Termite mounds (Fig. 1) were abundantly found in various locations within the Universiti Sains Malaysia campus. The collection site for this study was around 100 m away from our laboratory. Live termites were collected from soil mound and were transported to the

Fig. 1 Photograph of soil mound (collection site) of termite *M. carbonarius* located under a tree in the Universiti Sains Malaysia campus

laboratory along with mound material and were then separated into soldiers and workers based on their obvious morphological characteristics. Each termite was washed with sterile distilled water, dried on a filter paper and externally sterilized with 70% ethanol. Following the evaporation of ethanol, the entire gut was withdrawn with a sterile forceps and crushed. The entire procedure was carried out under sterile condition. The crushed guts were homogenized in 1 ml of sterile distilled water, centrifuged at 1,000 rpm for 1 min to remove large gut debris, and serially diluted. Aliquots of 0.3 ml were then plated on NA medium, followed by incubation at 30°C for 4 days. Colonies formed on the agar plates were picked and streaked on fresh NA plates for further purification. Pure bacterial isolates were obtained by subsequent streaking on fresh NA plates.

Screening of isolates for PHA producers

All pure isolates were screened for their PHA-producing ability according to the methods by Ostle and Holt (1982). Pure isolates grown on NA for 18 h were stained with Nile Blue A and observed under fluorescence microscope. The isolates were further confirmed for PHA accumulation by gas chromatography (GC) analysis as described by Braunegg et al. (1978). Among the PHA accumulating gut isolates, strain MC1 with rapid growth and higher PHA-producing ability was chosen for further characterization.

Gas chromatography analysis

Quantification of the amount of PHA in cells was carried out by GC analysis as described by Braunegg et al. (1978). At the end of the cultivation period, the cells were harvested by centrifugation (6,000*g*). The harvested cells were lyophilized and were subjected to methanolysis in screw-capped tubes at 100°C for 140 min in a mixture of 2 ml chloroform, 1.7 ml methanol, and 0.3 ml concentrated sulfuric acid. Upon completion, 1 ml of distilled water was added to the cooled mixture and vortexed for 1 min. After phase separation, the bottom layer (organic phase) was aspirated and the hydroxyl methyl esters thus prepared was analyzed by gas chromatograph (Shimadzu GC-2010 AF 230LV) equipped with a capillary column SPB-1 (30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) connected to a flame ionization detector. With nitrogen as carrier gas (1 ml/min), the sample (2 μl) in chloroform was injected using an auto injector (Shimadzu AOC-20i). The injector and detector temperatures were set at 270 and 280°C, respectively. The column temperature was increased from 70 to 280°C at 10°C/min.

Identification of *Bacillus* sp. MC1

API kit-50 CHB medium (Biomérieux Co.) and several morphological and biochemical tests like Gram staining, motility test, catalase, oxidase, methyl red, Voges-Proskauer test, growth on MacConkey agar, etc. were carried out for the identification of the *Bacillus* sp. MC1. Genomic DNA was isolated from *Bacillus* sp. MC1 as described by Wilson (1999). Universal primer set (URP): 5′-GTGCC AGCMGCCGCGGTAA-3′ and (UP2) 5′-GGGCCCCCGY CAATTCCTTTGARTTT-3′ were used for 16S rRNA gene amplification by PCR using the following conditions: Denaturation (1 min at 95°C), Annealing (45 s at 50°C) and Extension (1 min 30 s at 72°C) for 30 cycles followed by final extension step of 8 min at 72°C. PCR product was gel-eluted and purified using QIAEX® II Gel Extraction Kit (Qiagen) according to the manufacturer's instruction. The purified PCR products were then ligated into pGEM-T Easy Vector (Promega) and used for the transformation of *E. coli* JM 109 cells as described by Sambrook et al. (1989). Plasmids were isolated after the growth of cells and sent for sequencing at 1st BASE Laboratory (Malaysia). The 16S rRNA gene sequence was then analyzed using BioEdit Sequence Alignment Editor program and compared with other sequences available in the NCBI database.

Analysis of PHA accumulation by *Bacillus* sp. MC1 using various carbon sources

PHA biosynthesis studies in *Bacillus* sp. MC1 were carried out by both one-stage and two-stage cultivation methods. For one-stage cultivation, cells were first grown in 50 ml NB medium to reach an OD_{600} value of 3 and were used as the primary inoculum. 1.5 ml (3%) of the primary inoculum was transferred to 50 ml MSM in 250 ml shake flasks and cultivated on a rotary shaker at 150 rpm at 30°C. For two-stage cultivation, primary inoculum prepared by the above method was used to grow cells initially in 50 ml of NB (26 g l^{-1}) supplemented with 1% (w/v) of glucose for 20 h, before being harvested and transferred to 50 ml nitrogen-free MSM medium in 250 ml shake flasks and cultivated on a rotary shaker at 150 rpm at 30°C. In both the cultivation methods the following carbon sources: glucose, fructose, sodium acetate, sodium valerate and 1,4-butanediol were sterilized separately either by autoclave or by membrane filters (0.2 μm) and any one of these carbon sources were used at a concentration of 1% (w/v) in the PHA biosynthesis experiments. All the PHA biosynthesis experiments were carried out in triplicates and results documented as the average of three independent measurements. Twenty-five milligram of lyophilized cells were subjected to methanolysis at 100°C for 140 min in the presence of 15% (v/v) sulfuric acid to determine the PHA

content and composition. The resulting hydroxyacyl methyl esters were analyzed by GC as described by Braunegg et al. (1978).

Ultrastructural studies of *Bacillus* sp. MCI using transmission electron microscopy

Bacillus sp. MC1 cells from one-stage and two-stage cultivations incubated for 20 and 4 h, respectively were used for the morphological studies of the PHB granules under transmission electron microscope (TEM). Cells were harvested by centrifugation at 9,000 rpm for 10 min and were prepared for TEM studies as described by Kek et al. (2008). The sectioned specimens thus prepared were stained and observed using TEM at an acceleration voltage of 80 kV (Philips CM 12/STEM and JLM-2000FX11).

Ultrastructural studies of termite gut using transmission electron microscopy

Guts of major worker termites were withdrawn and immersed immediately in McDowell–Trump fixative (McDowell and Trump 1978) prepared in 0.1 M phosphate buffer (pH 7.2). The suspension was incubated at 4°C for 2 days. The guts were then directly subjected to dehydration by serial passage through 75, 95, 100 ethanol and 100% acetone. The dehydrated gut specimens were then prepared for TEM studies as described by Kek et al. (2008). The sectioned specimens thus prepared were stained and observed using TEM at a acceleration voltage of 80 kV (Philips CM 12/STEM and JLM-2000FX11).

Results and discussion

Isolation of PHA-producing bacteria from the termite gut

Several strains forming morphologically distinct colonies were isolated from guts of both worker and soldier termites. Among them, three strains designated as MC1, MC3 and MC4 were found to possess the ability to accumulate PHA granules, as observed by Nile blue A staining and GC analysis. Preliminary characterization showed all the three strains to be gram-positive and from the genus *Bacillus.* MC1 cells with rapid growth and higher PHA content was selected for further characterization.

Morphological and physiological characteristics of *Bacillus* sp. MC1

Bacillus sp. MC1 grown on NA at 30°C for 24 h formed round-shaped colony with smooth margin. The colonies were beige in colour with a diameter of 1–3 mm. Cell growth was optimum at 30°C, and no growth was seen when incubated at 4 and 55°C. Oxygen requirement growth test suggested the cells to be aerobic. Bright orange fluorescence with Nile blue A staining indicated the presence of PHA granules. The cells were rod-shaped, motile and often occurred singly or arranged in pairs when observed under phase contrast light microscope. The entire cell population sporulated within 24 h of cultivation. Selected taxonomical characteristics of *Bacillus* sp. MC1 are summarized in Table 1.

Identification of *Bacillus* sp. MC1

Amplification of 16S rRNA gene from the genomic DNA of strain MC1 using PCR gave an expected amplicon size

Table 1 Taxonomical characteristics of *Bacillus* sp. MC1

Characteristics	
Morphological characteristics	
Shape	Rod
Size (μm)	0.7–1.4 μm × 1.5–3.4 μm
Spore formation	+
Spore position	Central
Spore shape	Ellipsoidal
Gram staining	+
Motility	+
Parasporal crystal	−
Physiological characteristics	
Catalase	+
Oxidase	+
Cellulase	+
Voges-Proskauer	−
Methyl-red	−
Cultural characteristics	
Colony shape	Circular
Optimal pH	7
Optimal temperature	30°C
Growth on nutrient broth	+
Growth on nutrient agar	+
Growth on MacConkey agar	−
Utilization of	
Glycerol, ribose, D-xylose, L-arabinose	+
Galactose, glucose, fructose, sucrose	+
Mannitol, cellobiose, maltose, lactose	+
D-Arabinose, L-xylose, mannose, sorbose	−
Rhamnose, inositol, sorbitol, inulin	−
Arabitol, gluconate, fucose, xylitol	−

of 419 bp. The nucleotide sequence data reported in this paper appears in the GenBank nucleotide sequence database under accession number bankit 828999-*DQ 886480*. 16S rRNA gene sequence was compared with other respective sequences from the EMBL GenBank database using BLAST sequence-homology search. The sequence showed 99% homology with *Bacillus megaterium* and *Bacillus flexus* 16S rRNA gene sequences. The API kit-50 CHB results revealed strain MC1 to have 99% identity to *Bacillus megaterium*. *Bacillus* sp. MC1 strain isolated from the gut of *Macrotermes carbonarius* has been deposited at the Japan Collection of Microorganisms (MC1-JCM 14833).

Growth and PHA accumulation in *Bacillus* sp. MC1

The growth profiles of *Bacillus* sp. MC1 were studied with different NB media concentrations [13, 26 and 26 g l^{-1} supplemented with 1% (w/v) glucose] and LB medium to produce the highest biomass possible to be used in the first stage of the two-stage cultivation method for PHA biosynthesis. The growth profiles of *Bacillus* sp. MC1 showed maximum growth in NB (26 g l^{-1}) supplemented with 1% (w/v) glucose (Fig. 2). PHA accumulation was initiated in the early stages of growth as observed with Nile blue A staining. With such a pattern of growth and accumulation of PHA, the strain could be categorized as a growth associated PHA-producing bacterium along with other bacteria such as *Cupriavidus necator*, *Alcaligenes latus*, *Azotobacter vinelandii* and *Azotobacter beijerinkii* (Bormann et al. 1998). Growth after 20 h was seen to decrease slightly, probably contributing to the initiation of sporulation process in the cells.

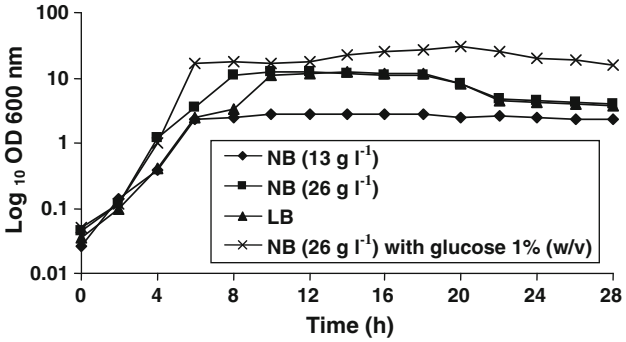

Fig. 2 Growth profiles of *Bacillus* sp. MC1 cultivated on LB and with different concentrations of NB medium

PHA biosynthesis by *Bacillus* sp. MC1 utilizing various carbon sources

One-stage cultivation method

One-stage cultivation method was employed to determine the ability of *Bacillus* sp. MC1 to grow and accumulate PHA simultaneously in a nutrient-limited condition. The strain was able to utilize all the five carbon sources tested for growth and PHA biosynthesis (Table 2). With glucose as the sole carbon source, *Bacillus* sp. MC1 was able to accumulate PHA to a peak of 20 wt% at 20 h of growth with a dry cell weight (DCW) of 1.53 g l^{-1}. GC analysis showed that the PHA consisted mainly of PHB homopolymers. Cell biomass was recorded maximum when grown on glucose compared to other carbon sources used in the one-stage cultivation. With fructose as sole carbon source, the PHA accumulation showed a different profile with PHA content reaching a maximum of 18 wt% after 8 h of growth and then decreased until no traces of PHA

Table 2 Biosynthesis of PHA by *Bacillus* sp. MC1 strain using various carbon sources by one-stage cultivation

Incubation time (h)	Carbon sources									
	Glucose		Fructose		Sodium acetate		Sodium valerate		1,4-Butanediol	
	PHA[a] content (wt%)	DCW (g l^{-1})	PHA[a] content (wt%)	DCW (g l^{-1})	PHA[a] content (wt%)	DCW (g l^{-1})	PHA[a] content (wt%)	DCW (g l^{-1})	PHA[a] content (wt%)	DCW (g l^{-1})
4	7.5	0.17	7.4	0.14	29.0	0.08	6.6	0.04	4.0	0.10
8	9.3	0.33	18.2	0.45	29.3	0.23	8.0	0.06	12.3	0.23
12	14.6	1.12	11.7	0.60	30.6	0.32	8.3	0.08	5.1	0.26
16	15.0	0.95	12.5	0.54	39.2	0.37	7.5	0.11	5.4	0.22
20	20.1	1.53	8.1	0.51	29.5	0.40	14.9	0.12	1.2	0.14
24	14.4	1.13	ND	0.33	25.3	0.45	12.4	0.12	1.0	0.11

Incubated at 30°C, 200 rpm and initial pH 7.0

ND not detectable

[a] PHA content in freeze-dried cells as analyzed by GC

was detected at 24 h of cultivation. The DCW was lower when grown on fructose compared to glucose. With sodium acetate, MC1 accumulated PHA to a maximal of 39 wt% after 16 h of growth. The PHA accumulation was relatively high when grown on sodium acetate compared to all other carbon sources used. However, DCW was lower compared to growth on glucose and fructose. Acetate is known to be one of the major end products of microbial fermentation in the gut (Slaytor 2000) and *Bacillus* sp. MC1 showed the maximum assimilation of this compound into PHB. In order to determine the ability of *Bacillus* sp. MC1 in accumulating PHA copolymers, sodium valerate was used as a sole carbon source. Sodium valerate has been used as precursor for the generation of 3-hydroxyvalerate (3HV) monomers in previous study (Doi et al. 1988). *Bacillus* sp. MC1 was able to assimilate sodium valerate and accumulate PHA to a maximal of around 15 wt% at 20 h of growth, but did not synthesise hydroxyvalerate monomers as observed by GC analysis. This could probably be due to absence of an active pathway involved in supplying of 3-hydroxyvalerate monomers. 1,4-Butanediol has been used as a precursor for the generation of 4-hydroxybutyrate (4HB) monomers in some PHA producers (Kunioka et al. 1989). When 1,4-butanediol was used as a sole carbon source, *Bacillus* sp. MC1 was able to accumulate PHA to a maximal of 12 wt% at 8 h cultivation with only 3HB as monomer units.

Two-stage cultivation method

Bacillus sp. MC1 strain was initially grown in NB (26 g l^{-1}) supplemented with 1% (w/v) of glucose for 20 h in the first stage to produce sufficient amount of cell biomass before transferring the cells to a nitrogen-free medium in the second stage to initiate PHA production. Cells do not multiply in second stage and therefore excess carbon source are utilized for PHA accumulation. As PHA biosynthesis in *Bacillus* sp. MC1 was growth associated, it was able to accumulate PHA in the first stage when grown on NB for 20 h. Hence at the beginning (0 h) of the second stage, cells contained up to 19 wt% of PHA (Table 3). Further PHA accumulation in second stage was observed in cultures grown on glucose and 1,4-butanediol as carbon source. With glucose, PHA accumulation was maximal at 34 wt% after 4 h cultivation period and then decreased on further cultivation. The latter indicated that the accumulated PHA was being mobilized even in the absence of external nitrogen-source. Accumulated PHA was composed of 3HB monomers as analysed by GC. With fructose, sodium valerate and sodium acetate as carbon source, cells did not show any further increase in PHA content in the second stage other than the PHA accumulated during the growth phase. PHA yield with sodium acetate was higher in the one-stage cultivation when compared to two-stage cultivation, inferring that acetate is more efficiently utilized for the biosynthesis of PHA in conditions where nitrogen-source is available. In nitrogen depleted condition, *Bacillus* sp. MC1 probably uses sodium acetate as an initial energy source for the energy-intensive sporulation process. Thus, limiting the amount of carbon source for PHA biosynthesis and thereby contributed to lower PHA accumulation. *Bacillus* sp. MC1 cells with 1,4-butanediol as sole carbon source accumulated higher amount of PHA (31 wt%) compared to that of one-stage cultivation. The accumulated PHA was observed to be slowly mobilized, which resulted in gradual decrease in PHA content from 31 wt% at 4 h to approximately 14 wt% at the end of 24 h incubation period. At the end of two-stage cultivation period, higher cell

Table 3 PHA biosynthesis by *Bacillus* sp. MC1 strain using various carbon sources by two-stage cultivation

Incubation time (h)	Carbon sources									
	Glucose		Fructose		Sodium acetate		Sodium valerate		1,4-Butanediol	
	PHA[a] content (wt%)	DCW (g l^{-1})	PHA[a] content (wt%)	DCW (g l^{-1})	PHA[a] content (wt%)	DCW (g l^{-1})	PHA[a] content (wt%)	DCW (g l^{-1})	PHA[a] content (wt%)	DCW (g l^{-1})
0	19.2	1.63	19.3	1.51	19.4	1.53	19.2	1.69	19.4	1.72
4	34.3	1.87	18.4	1.29	15.3	1.47	11.1	1.61	31.4	1.87
8	20.1	1.64	16.3	1.63	18.2	1.62	19.1	1.54	16.9	1.69
12	23.3	1.55	11.7	1.40	13.1	1.43	15.9	1.41	20.3	1.53
16	23.1	1.61	13.4	1.61	10.0	1.51	12.3	1.66	22.2	1.49
20	11.6	1.48	15.5	1.63	8.1	1.49	15.4	1.60	12.0	0.51
24	11.2	1.50	12.8	1.41	12.6	1.27	11.4	1.59	13.9	0.34

Incubated at 30°C, 200 rpm and initial pH 7.0

[a] PHA content in freeze-dried cells as analyzed by GC

biomass and PHA content was observed compared to one-stage cultivation with all the carbon sources used except for sodium acetate.

Ultrastructural studies of *Bacillus* sp. MC1 using transmission electron microscope (TEM)

When viewed under TEM, cells from the one-stage cultivation method were observed to contain numerous PHB granules. The granules were spherical and ranged from 0.15 to 0.7 μm in diameter and were seen to occupy almost the entire cell volume. Approximately 6–14 granules of variable sizes were observed in individual cell (Fig. 3a). Few cells from the one-stage cultivation were observed to contain spore localized at the cell terminal (Fig. 3b) but were not seen in the cell samples taken from two-stage cultivation. Pili were seen surrounding the MC1 cell surface (Fig. 3b). Images of cells from the two-stage cultivation revealed morphological changes in the granule. The shape of the granules within the cell varied at different stages of accumulation. Granules were seen to coalesce (Fig. 3c) and gradually lose its spherical shape. Tian et al. (2005) observed a similar phenomenon in intact cells of *Cupriavidus necator* H16 and had reported the coalescence of granules. Micrographs of dividing cells from the one-stage cultivation were observed to contain smaller sized PHB granules (Fig. 3d).

Ultrastructural studies of termite gut using transmission electron microscope

In situ morphology of gut microbiota in the paunch of the major worker *M. carbonarius* examined under TEM showed a vast region to contain numerous bacteria and at least seven distinct bacterial morphotypes was observed. Out of these distinct bacterial morphotypes two showed high possibilities of PHA accumulation. Morphotype 1 was a rod-shaped bacterium with smooth-wall containing electron-transparent inclusions in the cytoplasm (Fig. 4a). Electron-dense endospore 'e' was observed at the subterminal portion of morphotype 2 (Fig. 4b). Electron-transparent intracellular inclusions were also seen occupying the cytoplasm of this rod-shaped bacterium and the granules were observed to vary in shape and some were seen to coalesce (arrow). Morphotypes 1 and 2 showed high possibilities of accumulating PHA granules in their cell inferring that the conditions in the paunch were favourable for PHA accumulation. The morphology of morphotype 2 and the presence of endospore as well as small granules ascertain the presence of gram-positive bacterium from the

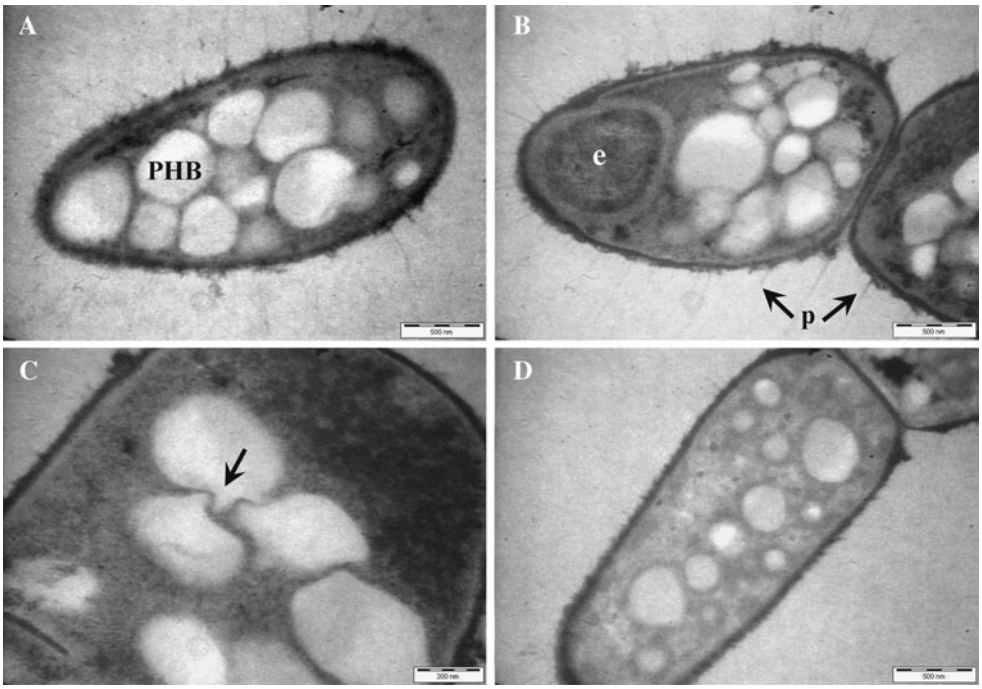

Fig. 3 Transmission electron micrographs of, **a** *Bacillus* sp. MC1 cell grown by one-stage cultivation method for 20 h. PHB granules appear as electron-transparent inclusions and around 12 granules can be seen occupying the entire cell. *Bar* 500 nm. **b** A *Bacillus* sp. MC1 cell with an endospore 'e' at the cell terminal and Pili 'p' surrounding the cell surface. *Bar* 500 nm. **c** *Bacillus* sp. MC1 cell grown by two-stage cultivation method for 4 h. The PHB granules are seen with protrusion that seems to interact with neighboring granules (*arrow*). *Bar* 200 nm. **d** A dividing *Bacillus* sp. MC1 cell with smaller sized PHB granules. *Bar* 500 nm

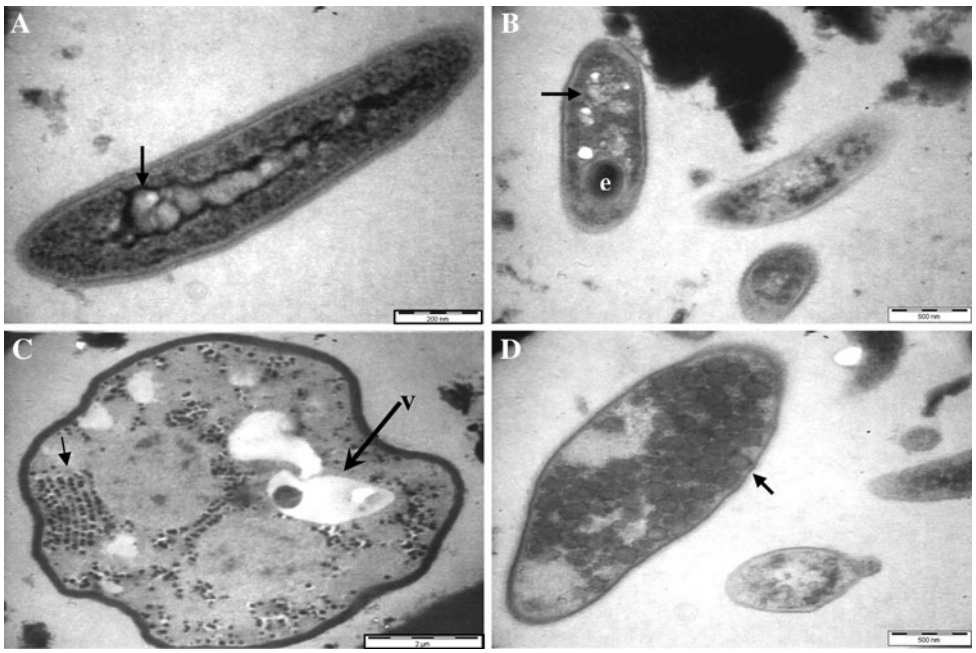

Fig. 4 Transmission electron micrographs of **a** Rod-shaped morphotype 1 with intracellular inclusions at the centre of the cell (*arrow*). *Bar* 200 nm. **b** Morphotype 2 with an endospore 'e' at the subterminal and the electron-transparent inclusions (*arrow*) in the cytoplasm of this rod-shaped bacterium. *Bar* 500 nm. **c** Morphotype 3 with an irregular shaped cell with thick and well-developed cell membrane. Two large vacuole-like structures 'v' and the ordered electron-dense elements (*arrowhead*) in certain regions of the cytoplasm can be observed. *Bar* 2 µm. **d** Morphotype 4 with a rod-shaped cell with slightly pointed ends. Cells containing spherical to oval-shaped structure (*arrow*) can be observed evenly distributed in the cytoplasm. *Bar* 500 nm

genus *Bacillus* in the worker gut. In addition, it further supports the evidence of sporulation process taking place in the gut. Morphotype 3 showed one of the most intriguing morphology and was the largest microorganism among the gut microbiota (Fig. 4c). The abundance and prominence of this microorganism in the gut as well as its irregular morphology was reflected in almost each section of the grid viewed under TEM. This morphotype possessed thick and well-developed outer membrane and electron-dense dark elements that were arranged in an orderly pattern in certain regions of the cytoplasm. Although morphotype 3 consisted of variable morphology, almost all cells contained large and irregular structures in the cytoplasm, which looked like vacuoles. Morphotype 4 was seen to contain numerous spherical to oval-shaped structures within the cell cytoplasm (Fig. 4d).

Previous studies have reported the presence of gram-positive bacteria from the order of Bacillales as part of the gut microflora (Thongaram et al. 2005; Wenzel et al. 2002). These studies suggest that the termite gut microenvironment favours the colonization of endospore-formers from the genus *Bacillus* sp. In the present study, PHA producing strain isolated from the termite gut had 99% homology with *Bacillus megaterium* and *Bacillus flexus* as observed from the API kit and 16S rRNA analysis results. Gram positive strains, especially from the genus *Bacillus* have been considered as sources for biomedically useful PHAs (Valappil et al. 2007). Unlike gram negative PHA producers such as *E. coli*, they are devoid of outer membrane lipopolysaccharide (LPS) endotoxins, which are known to co-purify with PHAs. LPS are pyrogens and induce a strong immunogenic reaction and hence considered unsuitable for biomedical applications (Chen and Wu 2005). PHA biosynthesis genes from *Bacillus* sp. have also been used for cloning and production of PHA in *E. coli* (Davis et al. 2008). In this study, *Bacillus* sp. MC1 was isolated from the gut of higher termites *Macrotermes carbonarius*, which feeds on dead plant litter. The microbial symbionts in the termite gut help in digesting the ingested lignocellulosic matter. *Bacillus* sp. MC1 strain was able to accumulate PHA utilizing various structurally unrelated carbon sources. Putative metabolic pathways for the biosynthesis of P(3HB) by this strain using different carbon sources used in this study has been described in Fig. 5. *Bacillus* sp. MC1 was also observed to grow well utilizing Carboxymethylcellulose (CMC) as sole carbon source on agar plates (results not shown). The ability of this gut isolate in utilizing lignocellulosic degradation products for PHB biosynthesis forms the base for our future research.

From the TEM studies, two distinct morphotypes showed PHA like granular structures in their cell, hence the

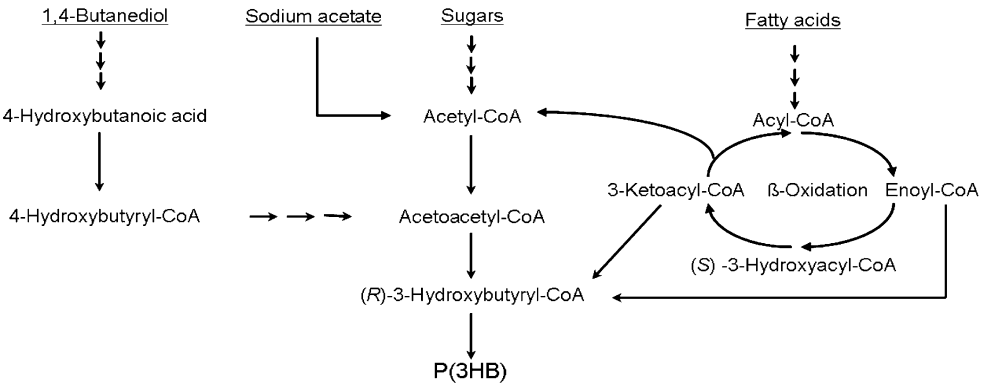

Fig. 5 Putative metabolic pathways for the biosynthesis of P(3HB) by *Bacillus* sp. MC1 utilizing various carbon sources (glucose, fructose, sodium valerate, sodium acetate and 1,4-butanediol)

possibility of occurrence of PHA accumulating bacteria from genera other than *Bacillus* cannot be ruled out. Termite gut microenvironment could thus be a potential source for isolation and screening of potent PHB producers with the ability to utilise inexpensive waste carbon source in the form of lignocellulosic degradation products. Although the close associations between termites and *Bacillus* are apparent from numerous studies, their specific role in the gut remains unknown. Studies on the PHB biosynthesis processes in these bacteria may provide additional insights into the physiological status of the termite gut microenvironment. This knowledge not only provides evidence for understanding termite-*Bacillus* co-evolution, but also projects termite gut as a good source for the isolation of potent strains for the development of novel processes in the conversion of lignocellulosic wastes into bioplastics in the future.

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