Identification of Thermophilic Bacterial Strains Producing Thermotolerant Hydrolytic Enzymes from Manure Compost

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Abstract Ten thermophilic bacterial strains were isolated from manure compost. Phylogenetic analysis based on 16S rRNA genes and biochemical characterization allowed identification of four different species belonging to four genera: *Geobacillus thermodenitrificans*, *Bacillus smithii*, *Ureibacillus suwonensis* and *Aneurinibacillus thermoaerophilus*. PCR-RFLP profiles of the 16S-ITS-23S rRNA region allowed us to distinguish two subgroups among the *G. thermodenitrificans* isolates. Isolates were screened for thermotolerant hydrolytic activities (60–65°C). Thermotolerant lipolytic activities were detected for *G. thermodenitrificans*, *A. thermoaerophilus* and *B. smithii*. Thermotolerant protease, α -amylase and xylanase activities were also observed in the *G. thermodenitrificans* group. These species represent a source of potential novel thermostable enzymes for industrial applications.

Keywords Manure compost · Thermophilic bacteria · 16S rDNA · Hydrolytic enzymes · Thermal stability

Introduction

Industrial enzymology is an important sector of biotechnology, characterised by a substantial growth over the last decade [1]. Hydrolytic enzymes have many industrial applications in the detergent sector, pulp and paper, and/or biofuel industry [2, 3]. An important source of thermostable enzymes can be found in the bacterial kingdom, where thermophilic prokaryotes are distributed among various groups in the phylogenetic tree of Bacterial and Archaeal domains [4].

Compost is a self-heating aerobic medium that results from microbial degradation of organic matter [5]. When the temperature reaches 50–55°C, the microbial thermophilic community becomes dominated by members of the genus *Bacillus* [6]. Investigation of 16S and 23S rRNA gene sequences has revealed a high heterogeneity in the genus *Bacillus* [7] and following extensive phylogenetic analysis, the genus has undergone major taxonomic revision leading to the establishment of new genera [8]. The genera, *Geobacillus* [9], *Ureibacillus* [10], and *Aneurinibacillus* [11] are outcome of this classification.

Here, we report on the identification and characterization of a cultivable thermophilic community in manure compost. Novel strains were identified and further investigated for thermotolerant hydrolytic activities.

Materials and Methods

Substrates and Chemicals

All substrates and chemicals were of reagent grade and were purchased from Sigma–Aldrich (Oakville, ON, Canada). All culture media were from DIFCO Laboratories,

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Becton, Dickinson and Company (Sparks, MD 21152 USA). Unless specified otherwise, all media and substrates were autoclaved at 121°C for 20 min. ABTS, Cu^{2+} , rhodamine B and sugars were filter-sterilized using 0.22 μm filters and added to the medium at 40°C.

Source of Microorganisms

Microorganisms were isolated from composted manures. The compost samples used for this research were produced by Fafard et Frères Ltée (St-Bonaventure, Qc, Canada).

Isolation and Culture of Thermophiles

Samples of compost 10% (w/v) (sample A: pH ~ 7.2, sample B: adjusted at pH 5.0) were enriched for 6 h at 60°C, then plated onto nutrient-agar plates at pH 7.0 and 5.0, respectively and incubated again at 60°C for 16 h. Compost sample C (pH ~ 7.2) was plated directly on nutrient-agar, without enrichment. Colonies from sample A, B and C were isolated on LB-agar plates at 60°C. Pure cultures were grown in Luria–Bertani broth, pH 7.0 (sample A), 5.0 (B) and 7.0 (C), respectively, at 60°C, with agitation (230 rpm) and preserved as suspension in 10% (v/v) glycerol at –80°C.

PCR, RFLP and Sequencing

Genomic DNA was used as template for PCR. 16S rRNA genes were amplified using the following primers: 27F: 5'-AGATTTGATCCTGGCTCAG-3' and 1522R: 5'-AAGGAGGTGATCCARCCGCA-3' [12]. The 16S-ITS-23S region was amplified using the following primer set: 27F: 5'-AGATTTGATCCTGGCTCAG-3' and 23S R 5'-CCCGCTTAGATGCTTTTCAGC-3' [13, 14]. PCR mixtures (50 μl) were prepared using 1 $\times$ Phusion BufferTM, 200 $\mu\text{mol l}^{-1}$ dNTP and 1.25 units high fidelity Phusion DNA polymerase (New England Biolab). For both 16S and 16S-ITS-23S regions, 0.4 $\mu\text{mol l}^{-1}$ of the respective primers was used. Initial denaturation was performed at 98°C for 30 s. Amplification was established in 30 cycles of 10 s at 98°C, 20 s at 55°C and 2.5 min at 72°C, with a final extension step at 72°C for 6 min. PCR products were purified using MinElute PCR purification kit (Qiagen). 16S rRNA genes were sequenced using an ABI Prism 3700 by the Biomolecular analysis platform (Université Laval). The 16S-ITS-23S regions (~5 kb) were digested with *EcoRI*, *HindIII*, *BamHI*/*EcoRI* endonucleases.

Analysis of 16S rRNA Gene Sequences

16S rRNA gene sequences were edited to start at the 5' CATGCA motif resulting in a final length of 1302 bp.

Searches for sequence similarity was performed using BLAST [15] using blastn accessed via the National Center for Biotechnology Information (NCBI) website. Additional 16S rRNA gene sequences were retrieved from GenBank database [16]. Multiple sequence alignments were performed with MEGA 4.1 [17] using CLUSTAL W [18] and a phylogenetic tree was inferred using the neighbour-joining method [19]. The evolutionary distances were computed using the maximum composite likelihood model, expressed as the number of base substitution per site. The stability of relationships was assessed by a bootstrap analysis based on 1,000 resampling of the neighbour-joining dataset [20].

Bacterial Characterization

Gram staining, oxidase, catalase, sugar metabolism profiles and nitrate utilization were determined for each isolate. For carbohydrate and nitrate utilization, isolates were cultured at 60°C using standard methods [21]. Durham fermentation tubes were used for detection of nitrogen gas resulting from denitrification.

Thermotolerant Hydrolytic Activity Detection

Enzymatic activity was detected on plates consisting of: Minimal medium (Mm): K_2HPO_4 , 2.5 g/l; KH_2PO_4 , 2.5 g/l; $(\text{NH}_4)_2\text{HPO}_4$, 1.0 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g/l; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/l; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0007 g/l; Casamino acids, 25% (w/v); and agar, 1.5% (w/v) supplemented respectively with the following substrates : glyceryl tributyrate 1% (v/v) or olive oil 1% (v/v) containing rhodamine B 0.001% (w/v) for detection of lipolytic activity [22], starch 1% (w/v) (amylase activity) [23], carboxymethylcellulose 1% (w/v) (cellulase and xylanase activity) [24], apple pectin 1% (w/v) (pectinase activity) [25], casein 1% (w/v) (protease activity) [26] and 2,2'-azino-bis(3-ethylbenzothiazoline) (ABTS) 6 mM and Cu^{2+} 300 μM (for laccase activity). All tests were carried out at 60°C. Each medium was buffered from pH 5–12 as indicated in the text, using the following buffers: 10 mM acetate buffer (pH 5 and 6), 10 mM Tris–HCl (pH 7–9) and 10 mM glycine–NaOH (pH 10–12).

Results and Discussion

Identification of Thermophiles

16S rRNA gene divergence among the isolates ranged from 0.4 to 13.9% forming four separate clusters corresponding

to four genera: *Geobacillus* (I), *Bacillus* (II), *Ureibacillus* (III) and *Aneurinibacillus* (IV). Phylogenetic relationships to previously cultivated species are shown in Fig. 1. The isolates resolved into four clusters (genera) and were related to *Geobacillus thermodenitrificans* (identities of 99.4–99.7% to strain NG80-2^T), *Bacillus smithii* (99.6% to strain DSM 4216^T), *Ureibacillus suwonensis* (99.2–99.3% to strain 6T19^T) and *Aneurinibacillus thermoaerophilus* (99.9% to strain DSM 10154^T), respectively. RFLP profiles (*EcoRI* and *HindIII*) for the 16S-ITS-23S region also clearly separated the isolates into four different clusters (Fig. 2). The 16S rDNA sequences data have been registered in the GenBank database under following accession numbers: CMB-A1 (GQ293453), CMB-A2 (GQ293454), CMB-A3 (GQ293455), CMB-A4 (GQ293459), CMB-A5 (GQ293460), CMB-A6 (GQ293461), CMB-A7 (GQ293456), CMB-A10 (GQ280379), CMB-B1 (GQ293457) and CMB-C1 (GQ293458).

Fig. 1 Neighbour-joining inferred tree based on 16S rRNA gene sequences showing the evolutionary relationship of isolates within previously characterized species. *Alicyclobacillus* was used as the 'outgroup'. Type strains are indicated by a T in *superscript*. GenBank accession numbers of 16S rDNA sequences are shown in *parentheses*. Bar length represent the number of base substitution per site. Bootstraps values expressed as percentage of 1,000 resamplings of the neighbour-joining dataset are showed at nodes. Bootstrap values less than 50% are not shown

Fig. 2 RFLP profiles of the 16S-ITS-23S region. Lane 1: CMB-A1; lane 2 (CMB-A2), lane 3 (CMB-A3), lane 4 (CMB-A10), lane 5 (CMB-A4), lane 6 (CMB-B1) and lane 7 (CMB-C1). M molecular weight marker (NEB 2 log DNA ladder, 0.1–10 kbp). Roman numerals (I, II, III and IV) indicate respective clusters. Left and right side of the marker shows *EcoRI* and *HindIII* restriction profiles, respectively

Table 1 Phenotypic and biochemical characteristics of thermophilic isolates

	I	I	I	I	I	I	I	II	III	III	III	III	IV
Characteristic	CMB-A1	CMB-A2	CMB-A3	CMB-A7	CMB-A10	CMB-B1	CMB-A4	CMB-A5	CMB-A6	CMB-C1			
Colonial Morphology	Rough	Rough	Smooth	Rough	Smooth	Glistening	n/a ⁱ	n/a ⁱ	n/a ⁱ	Rough			
Colony colour	Golden	Golden	Cream	Golden	Cream	White	White	White	Brown	White			
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods			
Gram stain	+	+	+	+	+	+	–	v	v	+			+
KOH (3%) test	–	–	–	–	–	–	–	–	–	–			–
Catalase activity	+	+	+	+	+	+	+	+	+	–			–
Oxydase activity	–	–	–	–	–	–	+	+	+	–			–
Denitrification	+	+	+	+	+	–	–	–	–	–			–
Growth temp range (°C)	45–65	50–65	50–65	50–60	50–65	45–65	50–60	50–60	50–65	45–60			
Growth pH range ^a	7–9.5	7–9.5	7–9.5	7–9.5	7–9.5	5–7.5	7–9.5	7	7–9	6–8			
L-Arabinose	+	+	–	+	–	–	–	–	–	–			–
D-Cellobiose	+	+	+	+	+	–	–	–	–	–			–
Lactose	–	–	+	+	+	–	–	–	–	–			–
D-Raffinose	–	–	–	w	–	–	–	–	–	–			–
D-Xylose	+	+	+	+	+	+	–	–	–	–			–
α-D-Sucrose	+	+	+	+	+	–	–	–	–	–			–
D-Sorbitol	–	–	–	–	–	–	–	–	–	–			–
D-Glucose	+	+	+	+	+	+	–	–	–	–			–
D-Galactose	+	+	+	+	+	–	–	–	–	–			–
D-Fructose	+	+	+	+	+	+	–	–	–	–			–

^a LB liquid medium*i* invasive, + positive reaction, – negative reaction, w weakly positive reaction, v variable reaction. Roman number I, II III and IV represent respective cluster

Characterisation of Thermophiles

Phenotypic characteristics for each isolate are listed in Table 1. Isolates from cluster I (*Geobacillus thermodenitrificans*) were able to carry out denitrification. Two different morphotypes were identified in the *G. thermodenitrificans* group: CMB-A1, CMB-A2 and CMB-A7 colonies were rough and slightly golden colour while CMB-A3 and CMB-A10 were smooth and creamy white colour (Table 1). Differences in sugar metabolism were also observed. Strains CMB-A1 and CMB-A2 used arabinose but did not use lactose, while CMB-A3 and CMB-A10 used lactose but did not use arabinose. These results are different from the strain *Bacillus thermodenitrificans* DSM 465^T which metabolizes both lactose and arabinose [27]. Sugar metabolism profiles for CMB-A1, CMB-A2 and CMB-A7 were closer to that previously reported for *G. thermodenitrificans* NG80-2 [28, 29] whereas CMB-A3 and CMB-A10 differed from NG80-2 in terms of utilization of arabinose. Furthermore, RFLP analysis revealed differences in the *EcoRI/BamHI* digestion pattern of the 16S-ITS-23S region within the *G. thermodenitrificans* isolates. Specifically, CMB-A3 and CMB-A10 lacked a 1.4 kb restriction fragment found in CMB-A1 and CMB-A2 (Fig. 3). Together, these features resolve these isolates into two groups in the *G. thermodenitrificans* cluster I, each group likely representing distinct strains of this species.

The isolate from cluster II identified as *Bacillus smithii* only utilized D-xylose, D-glucose and D-fructose. At variance, *Bacillus smithii* SC-J05-1 was reported to metabolize a large number of simple sugars [30].

Fig. 3 RFLP profiles of the 16S-ITS-23S region for *BamHI/EcoRI*. Lane 1 (CMB-A1), 2 (CMB-A2), 3 (CMB-A3), 4 (CMB-A10), M molecular weight marker (2 log DNA ladder)

Isolates from cluster III (CMB-A4 and CMB-A5) related to *U. suwonensis* formed colonies which spread over the surface of the plate. They did not metabolize any sugars tested in this study (Table 1), in agreement with previous results [31].

The remaining isolate from cluster IV, CMB-C1, was invasive and characterized by fractal growth. CMB-C1 did not metabolize any of the tested sugars, which is different from that reported for *A. thermoaerophilus* sp. nov., strain L420-91^T = DSM 10154^T which metabolizes D-glucose [32].

Hydrolytic Activities

Each isolate was screened for thermotolerant hydrolytic activities at 60°C. The Cluster I isolates related to *G. thermodenitrificans* (CMB-A1, A2, A3, A7 and A10) exhibited high extracellular esterase activity on tributyrin from pH 5.0 to 12.0 (see Table 2). These strains also showed some activity on olive oil from pH 6 to 9.5, as well as thermotolerant extracellular protease activity from pH 8.0 to 10.0 and α -amylase from pH 6.0 to 8.0. In contrast, the isolate from cluster IV, CMB-C1 related to *Aneurini-bacillus thermoaerophilus*, showed intense lipolytic activity on olive oil from pH 5–9. Isolate from cluster II CMB-B1 related to *Bacillus smithii*, also exhibited lipolytic activity on olive oil plates from pH 5.0 to 7.0 while isolates from cluster III, CMB-A5 and A6 related to *Ureibacillus suwonensis* exhibited no extracellular thermotolerant hydrolytic activity on any substrate tested here.

Lipases and esterases have been reported and characterized for different *Bacillus* species including: *G. stearothermophilus*, *G. thermoleovorans* and *G. kaustophilus*. However, no lipolytic enzymes have been reported as of yet for *G. thermodenitrificans*. Due to relatedness of CMB-A2 to strain NG80-2 (genome sequence ID: NC_009328), we identified genes that we used for designing primers. One of these genes coded for a lipase/esterase (GeneID: 4966646). We amplified this locus from CMB-A2, cloned, sequenced and functionally expressed the related gene in *E. coli*. Recently we reported on the detailed characterization of this enzyme (EstGtA2) from *G. thermodenitrificans* CMB-A2 [33]. We observed 99.1% identity with the respective enzyme sequence predicted in the genome of strain NG80-2. This result corroborates the relatedness of the isolates from cluster I to *G. thermodenitrificans*.

In a recent paper, a lipase produced by *A. thermoaerophilus* strain HZ was partly characterised [34]. The pH range reported for optimal lipase activity is compatible with the one reported here. Unfortunately, considering the conciseness of the phylogenetic analysis performed for strain HZ, one cannot ascertain its relatedness to the strain CMB-C1.

Table 2 Thermotolerant extracellular hydrolytic activities for isolates within each respective cluster (I–IV)

	I	I	I	I	I	II	III	III	III	IV
Hydrolytic activities	CMB-A1	CMB-A2	CMB-A3	CMB-A7	CMB-A10	CMB-B1	CMB-A4	CMB-A5	CMB-A6	CMB-C1
Lipolytic	5–9	5–9	5–9	5–9	5–9	5–9	–	–	–	5–9
Esterase	5–13	5–12	5–12	5–12	5–12	– ^b	– ^b	– ^b	– ^b	–
Amylase	6–8 ^a	6–7 ^a	6–8 ^a	6–8 ^a	6–7 ^a	–	–	–	–	– ^b
Xylanase	6 ^a	7–8 ^a	6 ^a	–	6 ^a	6 ^a	–	–	6 ^a	–
Cellulase	– ^b	– ^b	– ^b	– ^b	– ^b	–	– ^b	–	7 ^a	– ^b
Pectinase	– ^b	– ^b	– ^b	– ^b	– ^b	–	–	–	–	–
Protease	8–10	8–10	8–10	8–10	8–10	–	–	–	–	– ^b
Laccase	– ^b	– ^b	– ^b	– ^b	– ^b	–	–	–	–	–

^a Activity at 50°C^b Growth with substrate as sole carbon source

– No activity detected

Numbers show pH values where hydrolytic activity were detected at 60°C

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