Finore *et al. Chem. Biol. Technol. Agric. (2023) 10:9*
https://doi.org/10.1186/s40538-023-00379-7

Chemical and Biological Technologies in Agriculture

RESEARCH **Open Access**

Compost-derived thermophilic microorganisms producing glycoside hydrolase activities as new potential biocatalysts for sustainable processes

Ilaria Finore[1], Luigi Leone[1], Alessia Gioiello[1], Ida Romano[1], Silvana Cangemi[2], Pierluigi Mazzei[3], Alessandro Piccolo[2], Andrea Cattaneo[1], Barbara Nicolaus[1], Margarita Kambourova[4], Annarita Poli[1*] and Licia Lama[1]

Abstract

Background The management of the organic waste recycling process determines the interest in the thermophiles microorganisms involved in composting. Although many microbial enzymes have been isolated and studied for their industrial and commercial uses, there is still a continuous search for microorganisms which could synthesize industrially feasible enzymes, especially when the microbial diversity of cow dung itself makes a potential source of biotechnological enzymes.

Results The composting process studied at the Experimental Station of the University of Naples Federico II (Castel Volturno, Caserta, Italy) was characterized by fresh saw dust 40%, bovine manure 58%, and 2% mature compost as raw organic substrates, and its thermophilic phase exceeded a temperature of 55 °C for at least 5 days, thus achieving sanitation. Six microbial strains were isolated and designated as follow: CV1-1, CV1-2, CV2-1, CV2-2, CV2-3 and CV2-4. Based on 16S rRNA gene sequence, HRMAS–NMR spectroscopy, and biochemical investigations, they were ascribed to the genera *Geobacillus* and *Bacillus*. All the microbial isolates were qualitatively screened on plates for the presence of hydrolytic activities, and they were quantitatively screened in liquid for glycoside hydrolase enzymes in the extracellular, cell-bound, and cytosolic fractions. Based on these results, strains CV2-1 and CV2-3 were also quantitatively screened for the presence of cellulase and pectinase activities, and pH and temperature optimum plus thermostability of cellulase from CV2-1 were analyzed.

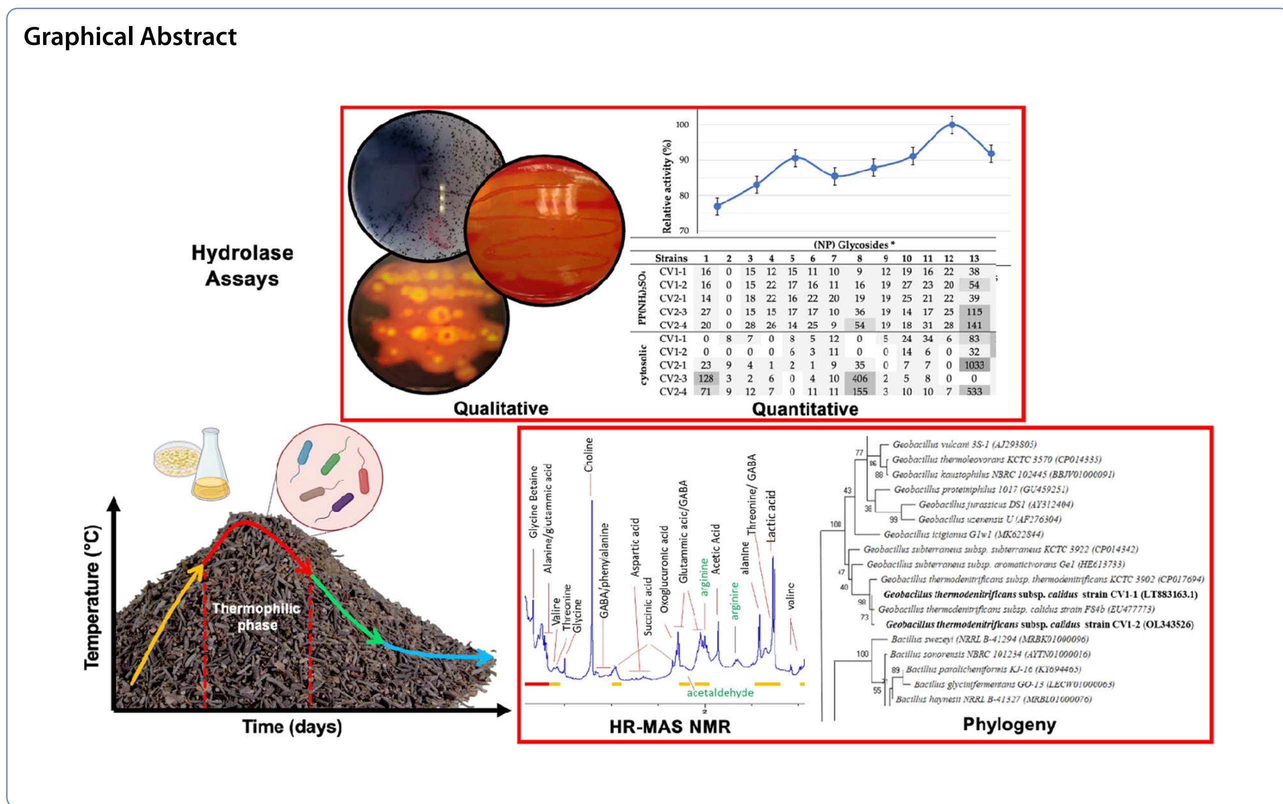
Conclusions The isolation and the identification of these thermophilic microorganisms such as *Geobacillus toebii*, *Geobacillus galactosidasius*, *Bacillus composti*, *Bacillus thermophilus* and *Aeribacillus composti* have allowed the study of the biodiversity of compost, with emphasis on their primary metabolome through an innovative and underutilized technique, that is HRMAS–NMR, also highlighting it as a novel approach to bacterial cell analysis. Subsequently, this study has permitted the identification of enzymatic activities able to degrade cellulose and other polymeric substrates, such as the one investigated from strain CV2-1, which could be interesting from an industrial and a biotechnological point of view, furthermore, increasing the knowledge for potential applicability in different industrial fields as an efficient and environmentally friendly technique.

Keywords Compost, Extremozyme, Thermophile, Glycoside hydrolase activities, ^{1}H–^{13}C HRMAS

*Correspondence:
Annarita Poli
apoli@icb.cnr.it
Full list of author information is available at the end of the article

© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

Graphical Abstract



Introduction

Composting consists of a self-heating and aerobic process in which organic waste materials are converted into humus-like substances through a degradation conducted by microorganisms and their enzymes.

Four main phases can be found in the composting process: (1) degradation of organic matter characterized by a rapid increase of temperature values (temperature range: 10–42 °C; duration: 24–72 h), (2) the thermophilic phase (45–70 °C; from several days to several weeks) characterized by the metabolic activities of endogenous microorganisms that are responsible of prolonged high temperature values, (3) the third phase is characterized by a reduction of the temperature and the re-establishment of the heat resistant microorganisms (65–50 °C; 1–2 months), and (4) the finishing-curing phase (50–23 °C; 1–4 months), in which occurs the stabilization produced organic matter [1–3].

However, knowledge of the microbiology of this process is limited, particularly concerning the thermophilic microorganisms associated with degradation of organic materials. Several novel species of thermophiles were isolated and characterized in different phases of the composting process, such as *Thermus thermophilus* [4], *Geobacillus toebii* [5], *Planifilum composti* [6], *Geobacillus galactosidasius* [7], *Geobacillus thermodenitrificans* and *Aneurinibacillus thermoaerophilus* [8] and *Aeribacillus composti* [9].

Thermophilic microorganisms (optimum growth temperature of 50 °C or above) are among the most studied extremophiles during the last four decades. They have attracted great attention, because they represent an underutilized and innovative source of novel thermostable enzymes, extensively studied for their use as biocatalysts in the food, pharmaceutical, chemical, biofuels, and textile industries. In fact, mesophilic enzymes have shown poor stability at high temperatures or at extreme pH values, limiting their actual application under industrial reaction settings. Biotechnological studies have, therefore, focused on extremozymes which have high stability in extreme conditions of temperature or pH, in the presence of organic solvents and at high ionic concentrations and are better suited for industrial conditions [10]. Therefore, due to their optimal activity and stability, extremozymes offer new catalytic alternatives for current industrial applications. They also represent the cornerstone for the development of environmentally friendly and sustainable industrial technologies exploiting their higher activity, stability, and robustness [11]. Many advances in industrial biocatalysts have been achieved in recent years. However, the potential of catalysis using extremozymes is far from being fully realized.

Thermophilic bacteria are a potentially rich source of hydrolases for biomass deconstruction. However, these bacteria generally secrete low levels of glycoside hydrolases, especially cellulolytic enzymes [12]. The search for new thermostable hydrolases with novel functions and appropriate performances is of much interest, even though hydrolases have already been employed in several industrial applications [13], providing additional benefits such as easy purification, higher stability, better productivity, enlarged process windows and conditions for more favorable thermodynamics such as a shift of reaction equilibrium [14]. These benefits make the identification and characterization of hydrolases from hot terrestrial environments attractive for developing various novel and improved processes in industry. Although, many microbial enzymes have been isolated and studied for their industrial and commercial uses, there is still a continuous search for microorganisms that can potentially synthesize industrially feasible enzymes, especially when microbial diversity of cow dung makes it a potential source for the said purpose.

Considering an increasing demand for enzymes, such as glycohydrolyses, proteases, polymerases, nucleases, and lipases, it is becoming extremely difficult to ignore the importance of hydrolytic enzymes as potential biocatalysts in a wide variety of industries, including chemical processing, dairy, agrochemicals, paper, cosmetics, pharmaceuticals, surfactants, detergents, polymers, and biofuel synthesis. A wide number of enzymes from thermophilic microorganisms have been characterized, such as cellulases, amylases, pullulanases, xylanases, mannanases, pectinases, chitinases, proteases, lipases, esterases, and phytases [15]. In particular, cellulases, hemicellulases, and xylanases have had important applications in the bleaching of paper and in environmental decontamination [16]. Today, biodetergents possess enzymes such as amylase, protease, cellulase, and lipase that are resistant to washing conditions. Additionally, thermozymes have been used in the creation of optical nanosensors and analytes creating innovation in developing nano-biomedicines and sensoristics [17].

Moreover, thermophilic bacteria isolated from compost capable to convert lignocellulosic biomass to obtain mono- and oligosaccharides, have also been described. They belonged to *Bacillus* species, such as *B.* clade *subtilis* [18], isolated from raw composting materials. The study of cellulolytic activity of these microorganisms was performed highlighting their potential for cellulose conversion and subsequently, for second generation bioethanol production that represents one of the best alternatives to the fossil fuels [19]. Recently, López et al. [20] described the characterization of thermophilic lignocellulolytic microorganisms in composting process of mixed shredded tomato plant waste (stalks and leaves) with pine chips. The studies revealed that while the complete fungal thermophilic population was characterized by lignocellulose-degrading enzymes, instead only 8–10% of the thermophilic bacteria exhibited them, and in particular enzymatic activities regarding the hemicellulose degradation. The xylanolytic bacterial isolates belonged mainly to the Firmicutes phylum (96%), with few representatives belonging to Actinobacteria (2%) and Proteobacteria (2%). In particular, the majority of the isolated species have been identified as *Bacillus licheniformis* and *Aeribacillus pallidus,* in addition to strains of *Caldibacillus thermoamylovorans* [18] and *Geobacillus thermodenitrificans,* that exhibited considerable enzyme activities. Since cellulose is the main polysaccharide in plant biomass, while xylans, β-glucans and mannans are the most important constituents of hemicellulose in plant cell walls, the determination of the activities of cellulase, xylanase, β-glucanase and mannanase can indicate the cellulolytic and hemicellulolytic degradation potential of a microorganism.

The aim of this work was the isolation of new thermophilic microorganisms, from composting plant of the Experimental Station, University of Naples Federico II at Castel Volturno (Caserta, Italy), capable to digest and to valorize polymeric waste biomass, such as cellulose, xylan and starch. In this study, a culture-dependent approach was employed to proceed with the isolation of the single species and exploit the applicative potential of microorganisms and their active biomolecules. The starting material for bacterial isolation was represented by compost which contained manly bovine manure (58%). The molecular composition and the metabolomic fingerprint of isolated species was innovatively identified by the HRMAS NMR spectroscopy through a semisolid state analysis. This technique was combined with 16S rRNA gene sequencing to determine the phylogenetic trees. To identify enzymatic activities able to degrade cellulose and other polymeric substrates, a biochemical characterization was performed: all the microbial isolates were qualitatively screened on plates for the presence of hydrolytic activities, and they were quantitatively screened in liquid for glycoside hydrolase enzymes in the extracellular, cell-bound, and cytosolic fractions.

Materials and methods

Composting site

Composting processes have been carried out at the composting plant of the Experimental Station of the University of Naples Federico II at Castel Volturno (Caserta, 3′ N 13° 59′ E, Campania region, Italy) by mixing raw organic materials (saw dust 40%, bovine manure 58%, mature compost as starter 2%). The choice of material to

be delivered to the plant was determined after a feasibility study based on the availability of agricultural residues present in the company and in the territory surrounding. The presence of livestock farms and buffaloes (related to milk and mozzarella cheese production, typical of Campania region) has, therefore, oriented the choice toward a farm composting system (on-farm), based on the intake of manure from surrounding farms, mixed with farm residues, mainly from maize cultivation. The process was carried out under shed with covered surface of 400 m^2 and it was obtained through 45 day on-farm composting (active or thermophilic phase) with forced aeration of static piles, followed by a 2 month curing period. In the composting system, assembled within farming facilities, a forced air distribution system with rotary pump and perforated pipes made of polyethylene, 10 m long and 0.15 m in diameter was temperature-controlled and assured by air injection through a basal net of tubes connected to a blower. The temperatures and the air requirements are automatically controlled by a control unit and a data-logger system with probes for continuous recording temperature, humidity and oxygen levels inside the heaps. Pile wetting was done through an irrigation system, manually activated when it was gravimetrically determined that the relative humidity (RH) was < 50%. Composting temperatures were measured by PT100 thermo-sensors placed in the core of pile. During the thermophilic phase, pile heating has exceeded 55 °C for at least 5 days, to achieve disinfection. Composting pile samples on the 15th day from the start of the process (temperature of 43.6 °C) were named "1CV", while composting samples collected on the 30th day from the start of the process (temperature of 58.8 °C) were named "2CV" and they represented the starting material for microbial isolation by culture-dependent approach (Fig. 1).

Microbial isolation by culture-dependent approach

One wet gram of each compost samples, 1CV and 2CV, were separately dissolved in 200 ml sterilized 50 mM phosphate buffer (pH 7.0) with agitation (120 rpm) for 24 h, temperature of 60 °C. The suspensions obtained were used to inoculate 10 ml of growth medium A containing (g/l): peptone, 8 g; yeast extract, 4 g and NaCl, 2 g. After 48 h at 60 °C, the microbial growth occurred and 100 µl, in duplicate, were used as an inoculum for the same solid medium, obtained by adding agar (Oxoid™) (1.8%, w/v). After 48 h incubation at 60 °C the colonies that appeared have been isolated and purified through the method of the serial dilution-plating, by means of re-streaking on new petri dish containing the same solid medium. Sub-culturing was conducted in Trypton Soy Broth-TSB (Oxoid) for 24 h at 60 °C and maintained as glycerol (Oxoid) stocks at − 20 °C for further study. To determine the purity of the isolates, the cell shape has

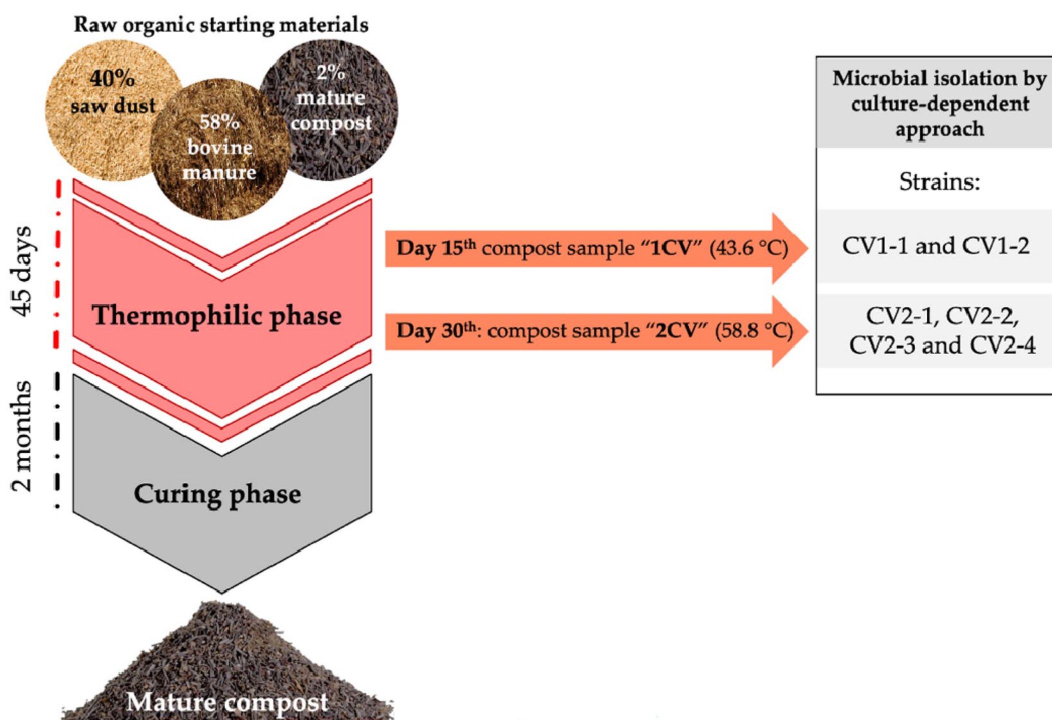


Fig. 1 Composting scheme and experimental design of microbial isolation of thermophilic microorganisms

been studied under phase-contrast microscopy (Nikon Eclipse E400, Nikon Europe, Badhoevedorp, The Netherlands), while the homogeneity of the colonies has been analysed under a stereomicroscope (M8, Leica, Leica Microsystems, Mannheim, Germany). A fraction of isolates was freeze-dried and stored until the NMR analysis.

Growth and morphologic characteristics

To define the optimal culture conditions of each isolate, growth ranges and optima of salinity, pH, and temperature were determined in a liquid medium A under aerobic conditions. The NaCl requirement for growth was assayed in a medium A containing various NaCl concentrations (0%, 0.5%, 1%, 5%, 7.5%, 10%, 15% and 20%, w/v). The pH and temperature range for growth was determined by incubating microorganisms at various pH values (5–10) and temperature values (40–80 °C), respectively. Growth was determined by measuring culture turbidity at λ 540 nm. The morphological characteristics of strains CV1-1, CV1-2, CV2-1, CV2-2, CV2-3, and CV2-4 were investigated using cultures grown on Trypton Soy Agar (TSA) (Oxoid) medium, at 50 °C for 24 h and observed using phase-contrast microscopy (Nikon Eclipse E400). Colony morphology was analyzed on a solid medium by stereomicroscopy (M8, Leica).

Strain biochemical characteristics

All biochemical tests were performed at the optimal growth temperature pH and NaCl concentration. Gram-staining was performed according to Halebian et al. [21]. Oxidase activity was determined by assessing the oxidation of tetramethyl-*p*-phenylenediamine and catalase activity was determined by assessing bubble production in a 3% (v/v) hydrogen peroxide solution [7, 9].

Phylogeny

For the DNA analysis, the collection of the cells has been performed through centrifugation at 5000 rcf for 15 min after 24 h of incubation at a temperature of 60 °C. The isolation of genomic DNA has been done employing DNAzol (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. The genomic DNA amplification through polymerase chain reaction (PCR) has been performed in a 50 µl PCR reaction mixture containing: 100 ng genomic DNA, 5 µl PCR buffer 10×, 2 mM $MgCl_2$, 200 µM dNTPs, 2 units of Platinum® Taq DNA polymerase (Invitrogen), 0.25 µM for each 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1517R (5′-ACGGCTACCTTGTTACGACTT-3′) and H_2O to 50 µl. The mixtures were amplified in a thermocycler iCycler® (Bio Rad). The amplification profile consisted of an initial denaturation of 3 min at 94 °C and 30 cycles of 30 s at 94 °C, annealing for 30 s at 55 °C and elongation for 1 min at 72 °C. A final extension step of 7 min was carried out at 72 °C. PCR products (5 µl) were electrophoresed on 2% agarose gel [22–24]. The 16S rRNA gene sequences of strains CV1-1, CV1-2, CV2-1, CV2-3, and CV2-4 were deposited in Gen-Bank/EMBL/DDBJ under the following accession numbers: LT883163.1, OL343526, OL343527, OL348060 and OL343528, respectively. The EzTaxon-e server (https://www.ezbiocloud.net) has been used to analyze the nucleotide sequences of 16S rRNA genes, and it has also been used to determine the values for the pairwise 16S rRNA gene sequence similarities between the closest species [25]. The software package MEGA X (version 10.1 https://www.megasoftware.net/show_eua) has been used to reconstruct the phylogenetic trees after multiple alignments of the data using CLUSTAL_X [26].

Distances (distance options according to Kimura's two-parameter model) and clustering were based on the neighbour-joining and maximum-likelihood algorithms [27–29]. Tree topologies were re-examined by the bootstrap method of resampling [30] using 1000 replications, and next to the branches it has been showed the percentage of trees in which the associated taxa clustered together. The initial tree(s) for the heuristic search were obtained automatically through the application of the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances, previously estimated using the maximum composite-likelihood (MCL) approach [31], and then selecting the topology with the superior log-likelihood value.

NMR analysis of thermophilic strains isolated from compost

The HRMAS NMR spectroscopy was applied to analyse, in the semisolid state, the molecular composition of isolated species, and to identify their primary metabolome and metabolomic fingerprint.

Each type of freeze-dried isolate biomass (4 mg) was packed into a HRMAS NMR 4 mm zirconia rotor, fitted with a perforated Teflon insert, soaked with approximately 10 µl of a phosphate buffer (brought to pH 7.0) and sealed with a Kel-F cap (Rototech-Spintech GmbH, Griesheim, Germany). The rotor was spun at a rate of 5000 ± 2 Hz. All NMR experiments were conducted at 25 °C on a 400 MHz Avance magnet (Bruker Biospin, Rheinstetten, Germany), equipped with a ^{1}H–^{13}C HRMAS probe, working at ^{13}C and ^{1}H frequencies of 101.5 and 400.13 MHz, respectively. Sample metabolic profile was investigated via proton spectroscopy by conducting three types of ^{1}H experiments: (i) a conventional pulse technique (CONV); (ii) a Carr–Purcell–Meiboom–Gill (eCPMG) pulse sequence based on a T_2 filter, enabling the selective suppression of short

spin–spin relaxing compounds; (iii) a diffusion-based pulse sequence, enabling the exclusive preservation of proton signals of relatively larger molecules (eDIFF). The three mono-dimensional ^{1}H experiments were conducted by setting a 90-pulse length ranging between 5.8 and 9.1 μs and 2 s of thermal equilibrium. Respectively, the spectral width and the number of scans were set to 32k and 200, for exp i and ii, and 16k and 256, for exp iii. eCPMG experiments were carried out by applying a total spin-echo time of 800 ms (based on cyclic *n*-repetitions of 2 ms single spin-echoes); eDIFF experiments were conducted through a stimulated echo pulse sequence and setting bipolar gradients (2 ms long sine-shaped pulses), a diffusion time of 200 ms and reaching 32.030 G m^{-1} as maximal gradient strength. In all cases the aqueous solvent signal was suppressed through on-resonance presaturation technique (54–65 db of power level attenuation), for experiments i and ii, and through 3-9-19 watergate technique, for experiment iii.

Structural identification of compounds detected in microbial isolates was assessed by two-dimensional (2D) NMR experiments, such as homonuclear ^{1}H–^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), as well as heteronuclear ^{1}H–^{13}C heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC). All 2D experiments were acquired with a spectral width of 16 (6410.3 Hz) and 300 (30 186.8 Hz) ppm for ^{1}H and ^{13}C nuclei, respectively, and a time domain of 2048 points (F2) and 256 experiments (F1). Homonuclear 2D spectra were based on 16 dummy scans and 64 total transients. Additionally, a mixing time of 80 ms and a trim pulse length of 2500 ms were set for the TOCSY experiment. HSQC and HMBC heteronuclear experiments were acquired with 16 dummy scans, 80 total transients, and 0.5 μs of trim pulse length. The experiments were optimized by considering 145 and 6.5 Hz as the optimal ^{1}H–^{13}C short- and long-range J couplings, respectively.

Spectra were processed using both Bruker Topspin software (version 2.1, BrukerBiospin, Rheinstetten, Germany) and MNOVA software (version 9.0, Mestrelab Research, Santiago de Compostela, Spain). Phase and baseline corrections were applied to all mono- and bidimensional spectra. The apodization was conducted for all proton 1D spectra by applying a 1 Hz factor, in case of CONV and eCPMG spectra, and 4 Hz for eDiff. No zero filling was applied during Fourier Transformation (FT). ^{1}H and ^{13}C axes were calibrated by associating the center of the β-CH_2 glutamine signal to 2.14 and 27.3 ppm, respectively.

Enzyme activities

Fractionation of cell components

Culture broths were centrifuged through Beckman Coulter JA-14 centrifuge at 15,344 rcf for 40 min at 4 °C and cell-free supernatants were concentrated with 80% $(NH_4)_2SO_4$ and then dialyzed against 50 mM TRIS–HCl pH 7.0. Cells were suspended in 50 mM TRIS–HCl pH 7.0 and treated with lysozyme (3 mg of lysozyme g^{-1} of wet cell) at temperature of 37 °C for 1 h, followed by ultrasonic treatment (Heat Systems Instrument) for 15 min, obtaining the cell homogenates. Cell extracts were separated by centrifugation through Hermle LaborTechnik Z 216 MK centrifuge at 3420 rcf for 40 min into cytosolic fractions (supernatants) and cell-wall fractions (pellets). The cell-wall fractions were suspended, treated with 0.5% Triton X-100 for 30 min at 4 °C under agitation, and centrifuged through Hermle LaborTechnik Z 216 MK centrifuge at 21,380 rcf for 40 min (cell-bound fractions). The three cellular compartments (extracellular partially purified with ammonium sulphate indicated by "PP$(NH_4)_2SO_4$", cytosolic and cell-bound fractions) were assayed for protein content using Bio-Rad protein assay kit with bovine serum albumin as standard protein according to Bradford [32].

Chromogenic substrate hydrolysis: screening of glycoside hydrolase (GH) activities

The compounds *p*-nitrophenyl β-D-xylopyranoside, *p*-nitrophenyl β-D-maltoside, *p*-nitrophenyl β-D-lactopyranoside, *p*-nitrophenyl β-D-cellobioside and *o*-nitrophenyl β-D-cellobioside, *p*-nitrophenyl β-D-glucopyranoside and *o*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-galactopyranoside and *o*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl α-L-arabinopyranoside, *p*-nitrophenyl α-L-arabinofuranoside, *p*-nitrophenyl α-D-maltoside, *p*-nitrophenyl α-D-glucopyranoside and lastly *p*-nitrophenyl α-D-galactopyranoside were used as colorimetric substrates for β-xylosidase (EC 3.2.1.37), β-maltosidase (EC 3.2.1.23), β-lactase (EC 3.2.1.108), exoglucanase (cellobiohydrolase) (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), β-galactosidase (EC 3.2.1.23), α-arabinopiranosidase (EC 3.2.1.55), α-arabinofuranosidase (EC 3.2.1.55), α-maltosidase (EC 3.2.1.20), α-glucosidase (EC 3.2.1.21), and lastly α-galactosidase (EC 3.2.1.22) enzymatic activities, respectively, and were purchased by Sigma-Aldrich.

After mixing 50 μl of substrate (100 mM solution) with 37 μg of protein sample (selecting the same and suitable protein amount of each different samples, to be able to set up the assay reaction in the desiderate final volume),

they have been suspended in 50 mM TRIS–HCl pH 7.0 (0.5 ml final volume), and the reactions have been incubated at 60 °C for 20 min. The release of the chromophore nitrophenol (NP) from the chromogenic substrates was checked by absorbance at λ 420 nm (ε 420 nm *p*-nitrophenol 15,000 M^{-1} cm^{-1}). One unit of activity was defined as the activity releasing 1 μmol of nitrophenol under the above assay conditions in 20 min [7, 25, 33]. Experiments were carried out in duplicate.

Qualitative enzymatic test on agar plates

All the isolates obtained from the compost sampling were screened for an evaluation of enzymatic activities of biotechnological interest. TSA medium containing 0.2% (w/v) starch was employed to verify the hydrolysis of starch using Lugol's iodine solution to flood cultures. Moreover, the hydrolysis of xylan and cellulose has been tested using 0.1% (w/v) Congo red (Sigma-Aldrich) to flood cultures, followed by the use of 1 M NaCl on TSA medium containing 0.2% (w/v) xylan or carboxymethylcellulose (CMC), respectively [34]. For proteinase and lipase detection, TSA medium plus 5.0% (w/v) skimmed milk and 1.0% (v/v) Tween 80 were used, respectively [9]. The presence of pectinase activity was assayed on agar plates containing polygalacturonic acid (0.2%, w/v) (ICN Biomedicals Inc.) as substrate. To verify the inducer effect of substrate, 100 μl of supernatant deriving from cultivation of strains (24 h) in TSB medium with and without polygalacturonic acid (0.2%, w/v), were disposed in the five millimeters wells bored into the agar plates. In addition, 100 μl of cell homogenate from a cultivation of 24 h in TSB medium were also tested. After incubation at 50 °C for 24 h, pH 7.0, the plates were stained with 10 ml of Ruthenium red solution (0.05%, w/v) for 20 min followed by washes with distillated water for 30 min. The presence of the clear halo around the wells indicated that the hydrolysis of specific substrates occurred suggesting the presence of enzymatic activities [35].

The presence of Gellan-lyase activity was assayed on hungry-agar plates containing (w/v) Sodium-Azide (0.01%), agar (1.5%) and gelzan (0.2%) (SIGMA) in H_2O. To verify the inducer effect of substrate, 100 μl of supernatant from cultivation of strains (24 h) in TSB medium with or without gelzan 0.2%, were disposed in the five millimeters wells bored into the agar plates containing gelzan (0.2%, w/v). On the other hand, 100 μl of intracellular fraction deriving from a cultivation of strains (24 h) in TSB medium at 50 °C, pH 7.0 were also investigated. Staining with Congo red (0.2%) was used as a qualitative method for test gellan-lyase activity showing a yellowish halo around the wells [36].

To test the ability of strains to degrade different substrates, 100 μl of extracellular and cell homogenates deriving from a cultivation of 24 h in TSB medium in the optimal conditions, were disposed in the five millimeters wells bored into the hungry-agar plates constituted of (w/v) 1.5% agar, 0.01% Sodium-Azide and 0.05% of the following Azurine cross-linked (AZCL) substrates (MEGAZYME): Arabinoxylan, Galactomannan, Curdlan, Arabinan, Dextran, Xyloglucan, Amylose, Cellulose, Galactan, β-Glucan, Chitosan, Pullulan, Collagen and Xylan. The plates were incubated for 72 h at 50 °C [37].

Quantitative enzymatic liquid test: cellulase, inulinase, pullulanase and gellan lyase

The activities were assayed using the supernatant deriving from a microbial growth of 24 h in TSB medium with and without the corresponding substrate carboxy methyl cellulose, inulin, pullulan and gelzan (0.2%) (ICN Biomedicals Inc.), respectively, as substrate and the raw cell homogenate from a microbial growth of 24 h in TSB medium at 50 °C, pH 7.0. The enzymatic assay was prepared as follows: 50 μl of 1% substrate in 50 mM TRIS–HCl pH 7 + 50 μl of sample. The reaction mixtures were incubated at 60 °C for 30 min on shaker (120 rpm) for all enzymes except for cellulase. Then, reactions were stopped by adding 100 μl of 3,5-dinitrosalicylic acid (DNS) then were placed in boiling water for 10 min. The absorbance was measured at λ 540 nm. The activities were expressed as Relative Activity (%) in appropriate incubation time [38]. The determination of the optimum pH for cytosolic cellulase from strain CV2-1 has been performed. The following buffers were employed glycine–HCl buffer (pH 3.0), acetate buffer (pH 3.5–4.0–4.6–5.0–5.6), phosphate buffer (pH 6.0–6.5), Tris–HCl (pH 7.0) and glycine–NaOH buffer (pH 8.6–9.0–10.0). For temperature optimum determination, the enzyme activity was carried out in 100 mM acetate buffer pH 4.6 at temperatures ranging from 35 to 105 °C.

The thermal stability determination of the cellulase enzyme from CV2-1 has been performed through the pre-incubation of the enzyme solutions in a temperature range 45–95 °C, also evaluating different time intervals; following the determination of the residual activities under standard assay conditions (at 55 °C), they have been expressed as percentage of the same enzyme tested without pre-incubation (100%). In detail, the enzyme was tested pre-incubating the solution at 45, 55, 85 and 95 °C for 2 h and at 45 and 55 °C for 24 h [39].

Results

Bacterial isolation from compost, growth, and morphologic characteristics

Bacterial strains designate with the following lab codes, by means of CV1-1, CV1-2, CV2-1, CV2-2, CV2-3 and CV2-4, represented the fastest growing microorganisms in medium A, and they were the only colony-forming strains at the highest dilutions. In particular, strains CV1-1 and CV1-2 were obtained from the sample named 1CV, while four strains, namely, CV2-1, CV2-2, CV2-3 and CV2-4 were isolated from the sample named 2CV. All isolates exhibited optimal growth in a medium TSB at 50 °C, with the exception of strain CV1-2 that grew at 60 °C (Fig. 2).

The optimal NaCl concentration requirement for the microbial growth was found (w/v) at 0.5% for all strains, as well as the optimal pH value of growth medium was found neutral. The isolates were all Gram-positive, rod-shaped and oxidase positive. Strains CV1-1 and CV1-2 showed creamy colonies with irregular edges, while CV2-1, CV2-3 and CV2-4 exhibited shiny, opaque, and white surface colonies, respectively (Additional file 1: Table S1).

Phylogeny of isolates from compost

The EzTaxon-e server (http://www.ezbiocloud.net/eztaxon) has been used to identify the bacterial isolates, and the 16S rRNA gene sequences, the gold standard for understanding the phylogeny of life, have allowed to strongly relate strains CV1-1 and CV1-2 to *Geobacillus thermodenitrificans* subsp. *calidus* (99.93% and 99.85% gene similarity, respectively); strains CV2-1, CV2-2, CV2-3 and CV2-4 were strongly related to *Bacillus licheniformis* (99.7%, 99.63%, 99.71% and 99.56% gene similarity, respectively). Based on 16S rRNA gene sequences, strains CV2-2 and CV2-3 showed 100% similarity (Additional file 1: Table S2); therefore, the further studies have concerned only the strain CV2-3 as it showed faster growth in both liquid broth and agar plate compared to strain CV2-2. The newly identified isolates were compared to appropriate type species within each genus of the phylogenetic trees using the maximum-likelihood algorithm (Fig. 3) or neighbor-joining algorithm (Additional file 1: Fig. S1), and analogous phylogenetic information regarding the isolates were obtained. All strains shared more than 97% identity with their closest phylogenetic relatives, thus suggesting that they could be considered at the same species level. Furthermore, strains CV1-1 and CV1-2 formed a clade with *Geobacillus thermodenitrificans* subsps. *calidus*, while strains CV2-1, CV2-3 and CV2-4 formed a clade with *Bacillus licheniformis* and *Bacillus aerius*.

NMR characterization of bacterial isolates

HRMAS NMR spectroscopy permitted to examine all bacterial isolates in the semi-solid state, after rehydration with a neutral phosphate buffer. Samples were evaluated

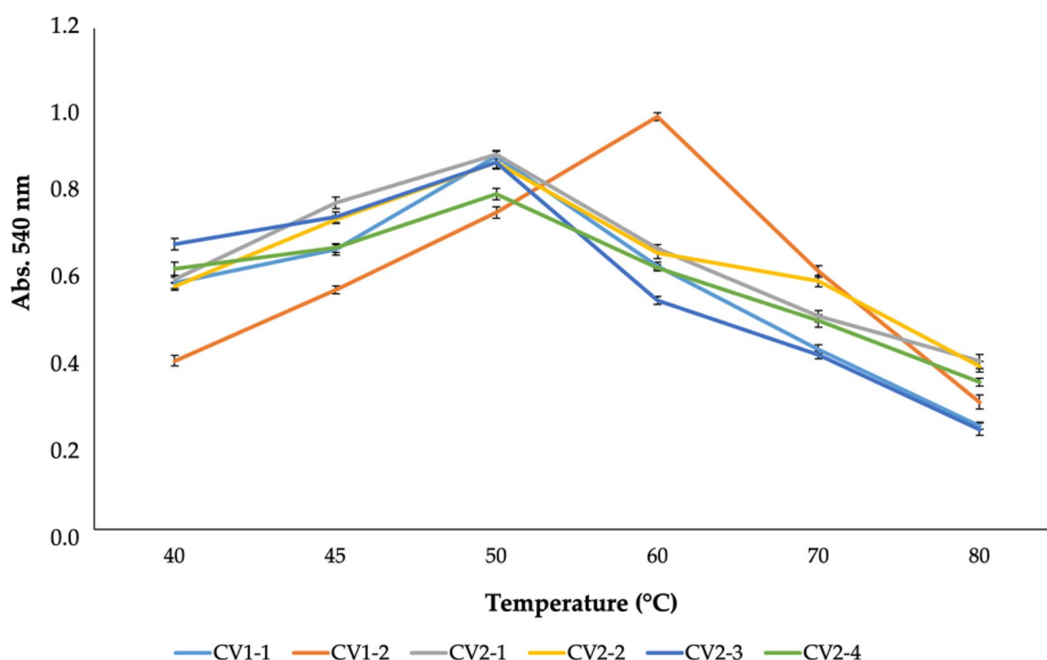


Fig. 2 Effect of temperature on growth of bacterial strains isolated from compost. The optical density was determined measuring the absorbance at *λ* 540 nm and the reported values represent the average of five determinations

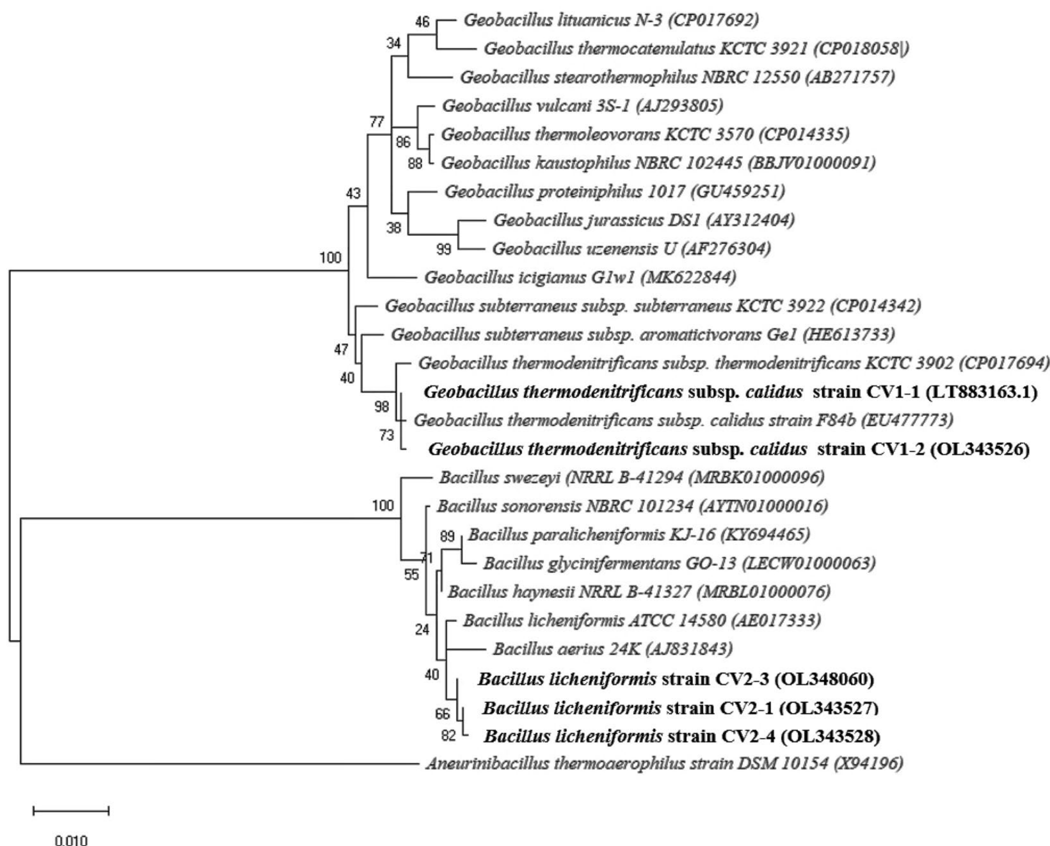


Fig. 3 Construction of the evolutionary phylogenetic tree has been done through the maximum-likelihood method, showing the relationship between the strains CV1-1, CV1-2, CV2-1, CV2-3 and CV2-4 and the related taxa based on partial 16S rRNA gene (Kimura 2-parameter method) with 1000 bootstrap replications. The scale represents a genetic distance of 0.010 nucleotide substitutions. The strains isolated in this work are reported in bold. *Aneurinibacillus thermoaerophilus* strain DSM 10154 (X94196) was used as an outgroup

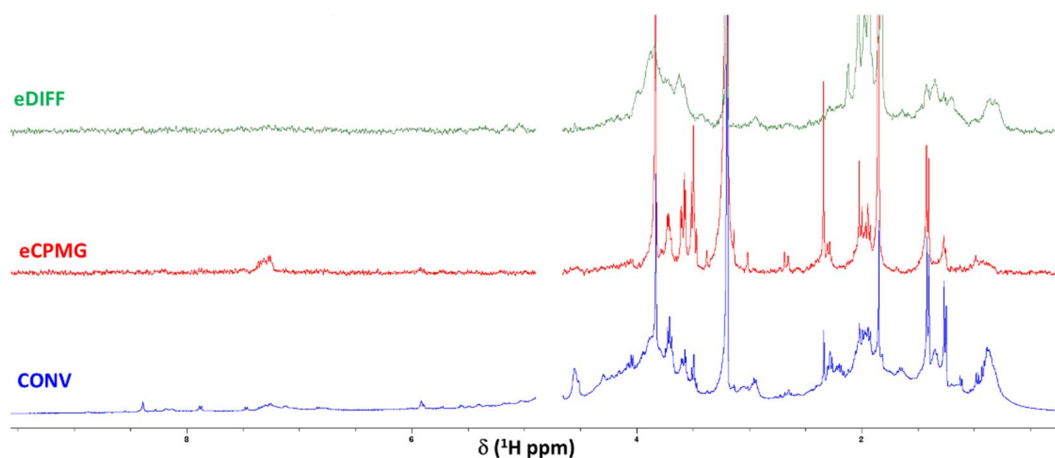


Fig. 4 ^{1}H-HRMAS–NMR Spectra of CV1-1 bacterial sample obtained by conventional pulse (CONV) and spectral editing techniques (eDIFF and eCPMG). In all cases, the distorted region of solvent suppression (4.85–4.65 ppm) was digitally removed from the figure

through conventional ^{1}H pulse technique (CONV), T_2-filtered Carr–Purcel–Meiboom–Gill NMR pulse sequence (eCPMG), and Diffusion-filtered Stimulated Echo sequence (eDIFF) (Fig. 4). While the former spectrum indiscriminately provided signals of all molecules present in the isolates, the last two techniques produced spectra deprived of signals deriving from large and small molecules, respectively. The experimental application of the last two edited pulse techniques to bacterial isolates was effective in showing signals which were totally or partially hidden in the conventional ^{1}H spectrum, due to peaks overlapping. This spectral clarification avoided to resort on possibly interfering deconvolution techniques, thereby allowing a more reliable compounds identification and a better semiquantitive signal evaluation.

The assignment of most signals in the ^{1}H spectra was reached on the basis of previous literature [40–43] and the analysis of 2D NMR spectra revealed ^{1}H–^{1}H and ^{1}H–^{13}C correlations (Additional file 1: Fig. S2). The identification of peaks detected in the representative ^{1}H CONV spectrum of strain CV2-1 is shown in Fig. 5. Remarkably, the assignment of compounds, such as arginine and acetaldehyde, was possible only via eCPMG technique (Additional file 1: Fig. S3) which simplified the spectra by removing the presence of overlapping signals, ascribable to slow-diffusing molecules. On the contrary, eDIFF spectra permitted to clearly and neatly isolate and observe the signals of slow-moving compounds, mostly ascribable to lipids and glycoproteins (Additional file 1: Fig. S4).

A comparison between CONV (Fig. 6, Additional file 1: Fig. S3), eCPMG (Additional file 1: Fig. S4) and eDIFF (Additional file 1: Fig. S5) spectra of the CV1 and CV2 series permitted to both explore their metabolic profile, at different molecular levels, and appreciate peculiar differences.

The conventional ^{1}H-HRMAS NMR technique (Fig. 6, Additional file 1: Fig. S3), that enables detection of mostly unalerted primary metabolome of bacterial isolates, was efficient in revealing a specific profile for each CV. First, it was detected a neat difference between CV1 and CV2 series, consisting in a larger content of both glutamic acid (signals at 2.05 and 2.35 ppm) and saccharidic moieties (region between 3.7 and 4.2 ppm and peaks at 4.5 and 5.3 ppm) in *Bacillus* species (Fig. 6, Additional file 1: Fig. S3). Conversely, both *Geobacillus thermodenitrificans* isolates exhibited a larger content in alanine (1.38 ppm), accompanied by a lower concentration of lactic acid (1.25 ppm). Moreover, by comparing the two *Geobacillus thermodenitrificans* isolates (CV1-1 and CV1-2), it was noted that the CV1-1 sample presented a much larger amount of choline, alanine and glycinbetaine than CV1-2. Interestingly, the HRMAS profiles of isolated thermophilic microorganisms, all identified as GRAM+, are very similar to those of bacteria Gram-positive isolated form alga laminaria digitata [43].

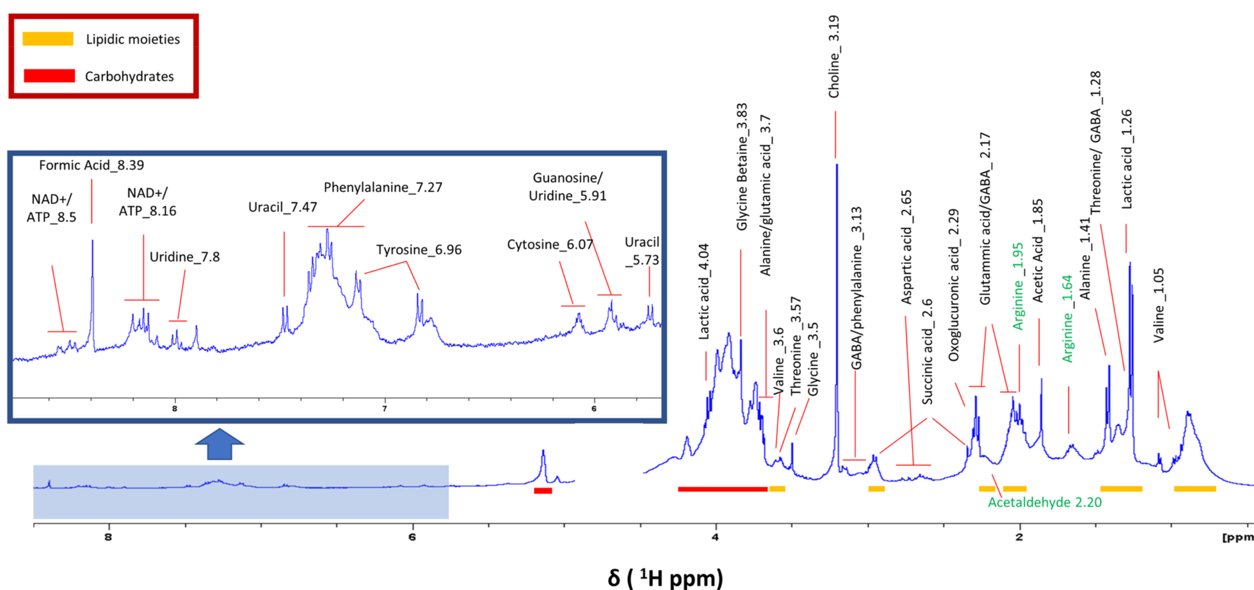


Fig. 5 Compound assignment to signals of an HRMAS–NMR ^{1}H conventional spectrum of strain CV2-1, where the values following the compound name refer to the central chemical shift (ppm). Compounds in green were assigned based on the eCPMG spectrum (Additional file 1: Fig. S4), while the peaks associated with coloured rectangles were isolated in the eDIFF spectrum and attributed to carbohydrates (red rectangles) and lipidic moieties (yellow rectangles) (Additional file 1: Fig. S5)

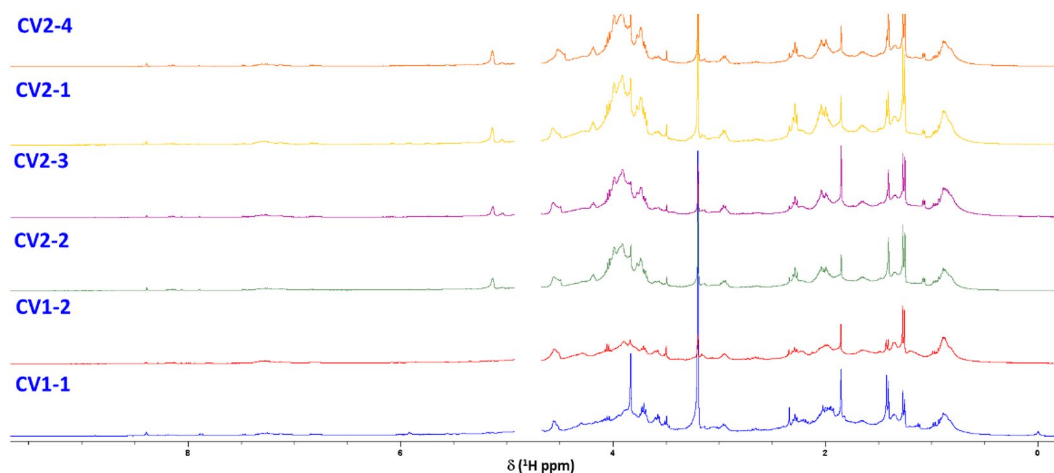


Fig. 6 ^{1}H HRMAS–NMR CONV spectra of bacterial species isolated from composting pile at the fifteenth (CV1-1 and CV1-2) and at thirtieth (CV2-1, CV2-2, CV2-3, CV2-4) day after the beginning of the composting process

The application of the eCPMG pulse technique produced simplified spectra (Additional file 1: Fig. S4) as compared to conventional ^{1}H spectra, by preserving only the peaks of low molecular weight compounds, including amino acids, organic acids, alcohols, and nucleotides. Their analysis not only confirmed findings for the ^{1}H conventional HRMAS spectra but also allowed to identify clearly other metabolites present in bacterial isolates, such as valine, acetylcholine, lactate, alanine, aspartate, and formic acids, which were significantly variable according to bacterial isolates (Additional file 1: Fig. S4; these specific peaks are highlighted with arrows in Additional file 1: Fig. S4). Remarkably, all of the eCPMG profiles of *Bacillus* series showed large similarity among them, except for CV2-3, that showed a slightly greater content of arginine and valine, and a smaller amount of formic acid. The eCPMG spectra of two *Geobacillus thermodenitrificans* were significantly different from those of bacillus series, while CV1-2 exhibited a more abundant content of lactic acid (peaks at 1.3 and 4.2 ppm) and a lower content of alanine. Similarly, CV1-1 presented much more intense signals in both the 1.7–2.2 ppm and 3.45–3.65 ppm regions.

Finally, the eDIFF spectra showed only signals pertaining to slow diffusing molecules, presumably of large molecular mass (Additional file 1: Fig. S5). Again, eDIFF spectra allowed to neatly differentiate CV1 from CV2 bacterial species, while, in line with eCPMG spectra, the profiles of all of CV2 series were very similar to each other. The eDIFF spectra of *Geobacillus thermodentrificans* revealed neat differences from those of Bacillus species (CV2 series), mostly due to a marked smaller abundance of carbohydrates in CV1 than CV2. Moreover, the profile of CV1-1 was significantly different from that of CV1-2 due to both the presence of much more intense signals in the alkyl region (2.3–1.8 ppm) and a very large content of choline (about 3.3 ppm, Additional file 1: Fig. S5).

Qualitative enzymatic tests of extracellular fractions

All isolates showed positive hydrolysis of starch and negative for polygalacturonic acid and Tween 80 degradation; all strains were negative for hydrolyses of AZCL-galactomannan, dextran, curdulan and chitosan, while only strains CV1-1 and CV1-2 were positive for xylan and pullulan degradation. Strains CV2-1, CV2-3 and CV2-4 were positive for protease, gelzan-lyase and cellulase activities; in addition, strain CV2-1 exhibited a degradation capability on pullulan and inulin (Table 1). Examples of enzymatic detection on agar plates are reported in Additional file 1: Fig. S6.

Quantitative enzymatic tests

Screening of glycoside hydrolase (GH) activities by chromogenic substrate

Glycoside hydrolase activities (U mg^{-1}) of the studied three cellular compartments are reported in Table 2.

Nitrophenyl glycosides have been used as substrates for enzymatic assays at temperature of 60 °C. After 20 min of incubation, the enzymatic solutions exhibited low amount of galactosidase activity, except for strain CV2-3 that showed an intermediate value of galactosidase, in both cytosolic and cell-bound fractions, when reacted on saccharidic moieties linked to NP through alpha bond; in addition, no reaction was produced in case of alpha bond linked to ring in ortho position; higher alpha galactosidase was detected in cell-bound enzymatic solution of strain CV2-1 (Table 2, entry 1–4). None of the tests performed revealed the presence of glucosidase activity

Table 1 Main biochemical characteristics of isolates (extracellular fractions). Hydrolysis of the selected polymers and AZCL-substrates on agar plate

		CV1-1	CV1-2	CV2-1	CV2-3	CV2-4
Polymers	Xylan					
	Starch					
	Carboxymethyl cellulose					
	Casein					
	Gelzan					
	Pullulan					
	Inulin					
AZCL- substrates	Arabinoxylan					
	Arabinan					
	Xyloglucan					
	Amylose					
	Cellulose					
	Galactan					
	β-Glucan					
	Pullulan					
	Collagen					
	Xylan					

Legend
Absence of hydrolysis (0%)
Very low hydrolysis (<25%)
Low hydrolysis (<50%)
High hydrolysis (>50%)
Complete hydrolysis (100%)

Table 2 Glycoside hydrolase (GH) activities (expressed as U mg^{-1}) detected through nitrophenyl glycosides in the extracellular ammonium-sulphate precipitates PP$(NH_4)_2SO_4$, cytosolic and cell-bound fractions of isolates

		Nitrophenyl Glycosides														
	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
PP$(NH_4)_2SO_4$	CV1-1	16	0	15	12	15	11	10	9	12	19	16	22	38	17	25
	CV1-2	16	0	15	22	17	16	11	16	19	27	23	20	54	19	26
	CV2-1	14	0	18	22	16	22	20	19	19	25	21	22	39	25	24
	CV2-3	27	0	15	15	17	17	10	36	19	14	17	25	115	19	24
	CV2-4	20	0	28	26	14	25	9	54	19	18	31	28	141	19	25
cytosolic	CV1-1	0	8	7	0	8	5	12	0	5	24	34	6	83	234	11
	CV1-2	0	0	0	0	6	3	11	0	0	14	6	0	32	118	0
	CV2-1	23	9	4	1	2	1	9	35	0	7	7	0	1033	10	0
	CV2-3	128	3	2	6	0	4	10	406	2	5	8	0	0	38	2
	CV2-4	71	9	12	7	0	11	11	155	3	10	10	7	533	9	0
cell-bound	CV1-1	16	8	24	15	15	12	8	27	0	57	78	47	163	0	33
	CV1-2	27	12	33	15	18	25	22	25	0	35	34	43	39	51	31
	CV2-1	598	10	22	5	11	22	28	1212	0	18	28	35	326	126	16
	CV2-3	224	0	20	1	5	9	0	1598	0	7	19	35	376	168	179
	CV2-4	30	0	28	8	11	14	4	105	0	16	27	38	175	0	74

Legend
Absence of activity (0 U)
Very low activity (<50 U)
Low activity (>50 U)
High activity (>100 U)
Very high activity (>1000 U)

Units of GH activities, expressed as μmol of *p*-NP released under the assay conditions. 1 = *p*-nitrophenyl α-D-galactopyranoside; 2 = *p*-nitrophenyl β-D-galactopyranoside; 3 = *o*-nitrophenyl α-D-galactopyranoside; 4 = *o*-nitrophenyl β-D-galactopyranoside; 5 = *p*-nitrophenyl α-D-glucopyranoside; 6 = *p*-nitrophenyl β-D-glucopyranoside; 7 = *o*-nitrophenyl β-D-glucopyranoside, 8 = *p*-nitrophenyl α-D-maltoside; 9 = *p*-nitrophenyl β-D-maltoside; 10 = *p*-nitrophenyl α-L-arabinofuranoside; 11 = *p*-nitrophenyl α-L-arabinopyranoside; 12 = *p*-nitrophenyl β-D-xylopyranoside; 13 = *p*-nitrophenyl β-D-cellobioside; 14 = *o*-nitrophenyl β-D-cellobioside; 15 = *p*-nitrophenyl β-D-lactopyranoside. All values are averages of two determinations.

(Table 2, entry 5–7). *B. licheniformis* strains showed an alpha-maltosidase activity in cytosolic and cell-bound fractions, particularly present in cell-bound samples of strains CV2-1 and CV2-3 (Table 2, entry 8, 9). Low alpha-arabinofuranosidase, alpha-arabinopiranosidase and beta-xylosidase activities were detected in all reactions (Table 2, entry 10–12), while, even if with different distribution among the various cellular compartments, all the strains have been shown to possess a beta-cellobiohydrolase with a preference for the beta bond connected to the ring in the para rather than ortho position (Table 2, entry 13, 14). Strains CV2-3 and CV2-4 showed a beta-lactase activity in cell-bound fraction (Table 2, entry 15).

Cellulase and pectinase activities of the selected strains CV2-1 and CV2-3

Since strain CV2-1 showed the highest cellobiohydrolase enzyme activity (Table 2) in cytosolic fraction (1033 total U mg^{-1}), further investigation of hydrolytic capability on carboxymethyl cellulose (reaction substrate) was carried out using the raw cell homogenate fraction of strain CV2-1. Carboxymethyl cellulose was selected as unique substrate to show the synergic actions of both cellobiohydrolase and endo-cellulase (here indicated as generic "cellulase"). Endo-cellulase presence was also revealed in the raw cell homogenate fraction of strain CV2-1 (data not shown). Cellulase enzyme of strain CV2-1 was active in a large range of pH values, between 3.0 and 9.0, with optimum at 4.6. The relative activities at pH 3.0 and 9.0 were about 87% and 40%, respectively, compared to the activity recorded at pH 4.6 (Fig. 7A). Cellulase was found to be active in a temperature range between 35 and 105 °C with maximal activity at 55 and 95 °C (Fig. 7B).

The thermal stability assay of cell-bound cellulase from strain CV2-1 was set up in 100 mM acetate buffer pH 4.6 and the enzyme reaction was incubated at 55 °C. For all tested conducted at 45 and 55 °C, no decrease of relative activity of cellulase was observed, except at 55 °C after 24 h of pre-incubation, in which a reduction of reducing sugars up to 97% was detected. Moreover, the relative cellulase activity, measured after 2 h of pre-incubation at 85 and 95 °C, was 89% and 82%, respectively.

Strains CV2-1 and CV2-3 showed pectinase activity in the cell homogenates fraction, measured with soluble polygalacturonic acid as a substrate in the reaction of enzymatic assay. The major activity was present in the cell homogenates of strain CV2-3 at 60 °C and pH 7. The pectinase activity of strain CV2-1 was 86% in comparison with CV2-3; in addition, the inulinase activity was predominant in the cell homogenates of strain CV2-1 at 60 °C and pH 7, and the activity of strain CV2-3 was 28% in comparison with CV2-1.

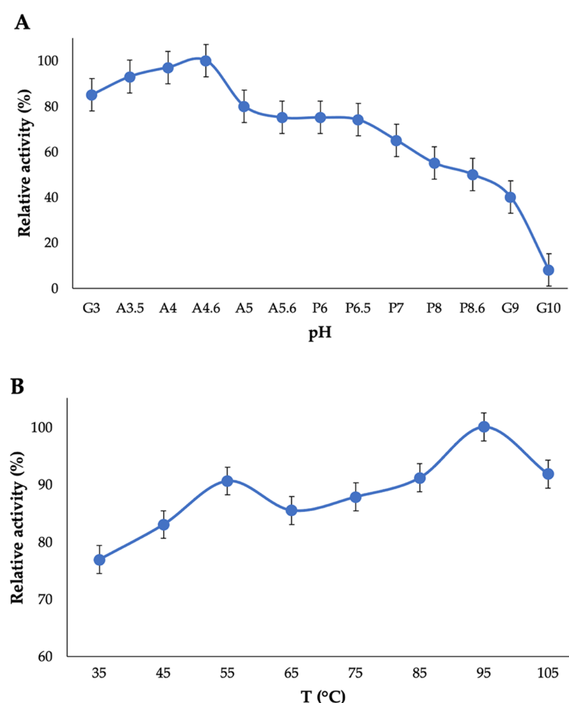


Fig. 7 pH (**A**) and temperature (**B**) curves of cellulase enzyme in the cell homogenate fraction of strain CV2-1. The values were expressed as Relative Activity (%). Buffer solutions employed: Glycine pH 3 (G3), Acetate pH 3.5 (A3.5), Acetate pH 4 (A4), Acetate pH 4.6 (A4.6), Acetate pH 5 (A5), Acetate pH 5.6 (A5.6), Phosphate pH 6 (P6); Phosphate pH 6.5 (P6.5), Phosphate pH 7 (P7), Phosphate pH 8 (P8), Phosphate pH 8.6 (P8.6), Glycine pH 9 (G9) and pH 10 (G10). The values after 1 h of incubation were expressed as relative activity (%) and they were subtracted from the contribution by the single sample and substrate at the time zero. The reported values are an average of three replicates and bars are the corresponding standard deviations

Discussion

Composting is a spontaneous process carried out by nature to convert complex substances into simpler substances available for plants. The main actors of the composting process are the thermophilic microorganisms, which efficiently operate the conversion of organic matter. This natural process, a part of generating fertilizer, commonly used in agriculture, is an interesting source for the study of the microorganisms which operate during this process. In fact, it is known that they produce biocatalysts capable of operating at high temperatures and in the presence of polluting substances; they are specialized and efficient biological systems. This manuscript reports the results deriving from the bacterial isolation from compost samples (collected during the thermophilic phase) produced in the experimental plant of Naples University Federico II located at Castel Volturno (Caserta, Italy), with the following mixture

composition: saw dust 40%, bovine manure 58%, mature compost as starter 2%. The isolated microorganisms were identified using a genetic approach based on 16S rRNA gene sequence, while their primary metabolome was determined through an HRMAS–NMR spectroscopic investigation.

This latter is a promising and still underutilized method to determine the metabolome of intact bacterial cells, identify specific strains and provide a more accurate snapshot of the actual physiological state of cells [41]. In this work HRMAS–NMR spectroscopy allowed to (i) identify, for the first time at the best of our knowledge, the primary metabolome of extremophilic microorganism isolated from compost, (ii) prove that the application of edited HRMAS spectra to intact cells enables a detailed molecular information of microbial species without resorting in any preliminary extraction, (iii) verify that the profile of Bacillus series was very different from that of *Geobacillus thermodentrificans*, and that each single isolate exhibited a diagnostic and peculiar metabolome, although at a relatively different extent. HRMAS represents a novel approach to bacterial cell analysis. It is fast and provides important information, which, in certain controlled conditions and microbial species, may even help to follow and understand processes involving their primary metabolome. Future HRMAS studies may lead to define microbial potentials without necessarily going through wet-lab experiments. Hopefully, by comparing the collection of metabolites of a species, one can prospect its ecological role or its biological activity/pathogenicity. This technique should prove a helpful tool in gene function validation, the study of pathogenesis mechanisms, the classification of microbial strains into functional/clinical groups, and the testing of bacterial molecules as performed here [44].

In addition, in this paper the compost was used as a starting material to study enzymatic activities able to convert lignocellulosic biomass wastes to obtain products with several potential biotechnological applications in different types of sectors. Therefore, these thermophilic strains were researched for enzymatic activities, such as cellulase, xylanase, pectinase and amylase which could have possible applications in industrial practice. For example, both the qualitative enzymatic test on agar plates and the quantitative enzymatic screening with chromogenic substrates highlighted the presence of interesting amylase enzyme in the strains CV2-1 and CV2-3, while they showed a cellulase activity for the strains CV2-1 and CV2-4. These results elucidated a microbial diversity of cow dung (as main components of composting mixture) as a potential source of microorganisms and their enzymes for biotechnological proposals.

The global enzyme market is proposed to reach USD 6.30 billion by 2023 at a Compound Annual Growth Rate (CAGR) of 5.8% from 2017 [44]. To enhance the efficiency of the composting process it is required to deeply comprehend the thermophilic microbial community involved in these biotransformations, which can be of particular importance to supply, at the same time, thermostable biomass-degrading enzymes for the biorefinery processes. Valorization of enzymes produced by thermophiles is one of the mail pillars of the "biorefinery" approach: in fact, in this context, waste is not considered material to be disposed of, but it can be used as a source of renewable energy and value-added chemicals [45, 46]. The enzymes produced by the new discovered thermophilic microorganisms will be used to degrade the lignocellulosic biomass obtaining monosaccharide units and oligosaccharides [34, 47]. The first, as they are fermentable sugars, could be used for biofuel production, while the oligosaccharides could be studied for their chemical and physical properties and biological activities and find employment in nutraceutical applications [48, 49]. One of the preferred environments for the recovery of lignocellulose-degrading microorganisms is exactly the composting processes [50].

Cellulase are the major industrially used enzymes, indeed it is reported that they occupy the third position in the world enzyme market [51]. The annual production of cellulose waste biomass is almost 4.0×10^7 units [52], and it represents an important source of renewable material. Liang et al. [53] reported that compost samples are a valuable font of cellulolytic thermostable enzymes. They differ from other hydrolases for their ability to produce glucose syrup through β1,4-glycosidic linkage breakage. Conventionally, the term "cellulase" is used to indicate an enzymatic system comprising three classes of enzymes, such as exoglucanases (EC 3.2.1.91), endoglucanase (EC 3.2.1.4), and β-glycosidase (EC 3.2.1.21). Among various microorganisms, bacteria are an appreciate source of cellulases thanks to their wide environmental adaptability, rapid generation time and high thermostability [54]. Many industrial applications are reported for cellulases synthetized by bacteria, fungi, and actinobacteria [55–57]. The most extensive studied microbial cellulases belong to the *Clostridium*, *Cellulomonas*, *Bacillus*, *Cellulovibrio*, *Thermomonospora* and *Paenibacillus* genera [58]. Recently, the attention of researchers is essentially focused on thermophilic microorganisms having a broader range of industrial applications, including bioconversion processes for production of molecules with high added value. In this paper, it has been described the presence of cell-bound cellulase fraction (raw fraction comprising synergic actions of both cellobiohydrolase

and endo-cellulase) produced by *Bacillus licheniformis* strain CV2-1; this fraction showed optimal activity at 55 and 95 °C (range 35–105 °C), suggesting that the release of reducing sugars monitored by DNS method was due to the simultaneous action of at least two enzymes, confirming its raw nature. Moreover, an optimum pH of 4.6 was detected, even if its pH range was between 3.0 and 9.0 offering a wide range for industrial purposes. In addition, thermal stability assays revealed the potential suitability of cellulase from strain CV2-1 for long time industrial applications at 45 and 55 °C; indeed, almost all activity was retained after 24 h of pre-incubation at both temperatures. Further thermal stability experiments, set up near to the other optimal working temperature, showed that this enzyme solution can greatly act on CMC, even after a pre-incubation at high temperatures such as 95 °C for 2 h; in fact, the measured residual activity was 82%. Therefore, the high temperature resistance kept by the cell-bound cellulase from *Bacillus licheniformis* strain CV2-1 makes it a good candidate for severe industrial processes.

Bacillus licheniformis JK7, isolated from the rumen of Korean goat, showed cellulolytic activity with an optimum of temperature for endoglucanase activity of 70 °C [59]. *Bacillus licheniformis* A5, isolated from liquor industrial production process, exhibited cellulase with pH optimum of 6.0 and temperature optimum of 50 °C and it retained 82% of its activity after 120 min at 80 °C [60]. On the other hand, several cases of cellulases obtained from *Bacillus* spp. isolated from compost have been reported in the literature [61–64]; however, they are predominantly extracellular cellulases, tested as crude enzyme, without any purification step (Table 3); moreover, to make a comparison between enzymatic activities it is necessary to also consider the activity of the enzymes itself, and this is made complex, because in the literature, most of the papers report their data as U mL^{-1}, without indications regarding the protein concentrations. Rather wide operating ranges of pH are reported: an activity greater than 40% is indicated about at pH range 3–10 for the protein precipitates $PP(NH_4)_2SO_4$ obtained from *Bacillus licheniformis* strain B7 and 2D55, and from *Bacillus* sp. DUSELR13. Regarding the temperature optimum, data in literature indicate 50, 60, 65 and 70 °C for *Bacillus licheniformis* strain B7, strain 380, strain 2D55 and for *Bacillus* sp. DUSELR13, respectively, with range of activity greater than 40% at ± 20 °C with respect to the optimum. Kazem et al. [56] reported that the isolation of cell-bound cellulases and xylanases from *Bacillus* sp. is a topic mostly not covered in the literature; additionally, in their work they found the highest cellulase activity in the extracellular fraction, while the cytosolic and the cell-bound activities showed a lower expression.

Therefore, this work provided an additional improvement in the clarification of the microbial role in the deconstruction of organic matter, and it has laid the foundations for setting-up controlled eco-sustainable bioconversion reactions. Composting of agricultural biomass waste fits in the research field of the quality of soil, crop protection, and plant health, enhanced by the need of a circular economy in an effort to exploit the most prominent compost-based formulations, also according to the demands of the market and to the government policies [65].

In addition, this research highlighted how enzymatic degradation, characterized by high yield, low energy consumption, and enhanced selectivity, could be the most efficient and environmentally friendly technique for converting complex lignocellulose polymers to fermentable

Table 3 Main biochemical properties of cellulases from *Bacillus* spp. isolated from compost

Strain	Cellulase localization	pH optimum and range of activity	T optimum and range of activity	Thermostability: residual activity (%) after pre-incubation at temperature (°C) for time (h)	Ref.
B. licheniformis CV2-1	Cell-bound	4.6 > 80%: 3.0–4.6 > 60%: 7.0–5.0	55 and 95 °C	~ 100% at 55 °C for 24 h 82% at 95 °C for 2 h	This work
B. licheniformis 380	Extracellular	7–9	60 °C	not determined	[61]
B. licheniformis B7	Extracellular	6–7 > 40%: ~ 3–10 > 80%: ~ 6–8	50 °C > 40%: ~ 30–70 °C > 80%: ~ 50–60 °C	not determined	[62]
B. licheniformis 2D55	Extracellular	7.5 > 40%: ~ 3–10.5 > 80%: ~ 4.5–9.5	65 °C	80% at 50–70 °C for 24 h	[63]
Bacillus sp. DUSELR13	Extracellular	5.0 > 40%: ~ 3–10 > 80%: ~ 4–8	75 °C > 40%: ~ 50–90 °C > 80%: ~ 65–85 °C	97% at 60 °C for 24 h 78% at 70 °C for 24 h	[64]

monosaccharides, and it is expected to make cellulases and xylanases the most demanded industrial enzymes [66]. In literature there are other examples of applications of functional bacteria derived from compost for industrial purposes and applied in a variety of different other productions. For instance, in the work by Tashiro et al. [67], the authors applied a bacteria consortium from marine–animal–resource composts rich in *Bacillus* strains on kitchen waste, an abundant and sustainable biomass resource, to produce L-lactic acid. A combination of the strains *Weizmannia coagulans* MN-07, *Caldibacillus thermoamylovorans* OM55-6, and *Caldibacillus hisashii* N-11 from marine–animal–resource composts in combination with a pH control strategy was used by Yue et al. [68] for the same final product, given the role of L-lactic acid as a precursor in the production of polylactic acid. Another interesting area of research applies the functional thermophilic bacteria isolated from compost to animals. This was discussed in the work by Inabu et al. [69], where the administration of the thermophile *Caldibacillus hisashii* to Japanese black calves contributed to an improve of growth performances, and it was also examined in the research by Miyamoto et al. [70], where the isolation of *Bacillus thermoamylovorans* and *Bacillus coagulans* from compost-fed mice suggested the potential of thermophilic probiotic bacteria derived from agricultural compost.

All these aspects combined, with the addiction of what it was highlighted by this same research, revealed the wide range of possibilities to enhance the scientific value of microbial systems as cell factories, which can be achieved through an in-dept analysis of their genomes and through the use of cloning to study high expression of their enzymatic activities.

Abbreviations

AZCL	Azurine cross-linked
CAGR	Compound annual growth rate
CMC	Carboxymethyl-cellulose
CONV	Conventional pulse
COSY	Correlation spectroscopy
DNS	3,5-Dinitrosalicylic acid
eCPMG	Carr–Purcell–Meiboom–Gill pulse
eDIFF	Diffusion-filtered stimulated echo pulse
GH	Glycoside hydrolase
HMBC	Heteronuclear multiple-bond correlation
HSQC	Heteronuclear single-quantum correlation
MCL	Maximum composite-likelihood
NP	Nitrophenol
PCR	Polymerase chain reaction
RH	Relative humidity
TOCSY	Total correlation spectroscopy
TSA	Trypton soy agar
TSB	Trypton soy broth
2D	Two-dimensional

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-023-00379-7.

Additional file 1: Figure S1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between isolated strains and related taxa. The scale represents a genetic distance of 0.010 nucleotide substitutions. The isolates obtained from this work are reported in bold. *Aneurinibacillus thermoaerophilus* strain DSM 10154 (X94196) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position. **Figure S2.** 2D NMR ^{1}H–^{1}H COSY (A) and ^{1}H–^{13}C HSQC (B) spectra of CV1-1 bacterial isolate. **Figure S3.** Edited HRMAS–NMR eCPMG spectra of bacterial isolates. Yellow arrows indicate signals visible in these spectra which were not detected in conventional ^{1}H-HRMAS–NMR spectra. **Figure S4.** Edited HRMAS–NMR eCPMG spectra of bacterial isolates. Yellow arrows highlight the signals whose presence and intensities changed significantly as a function of specific bacterial strain. **Figure S5.** Edited HRMAS–NMR eDIFF spectra of bacterial isolates. **Figure S6.** Qualitative enzyme activities detection on agar plates: a xylanase activity from strain CV1-1 revealed by Congo red; B amylase activity from strain CV1-2 revealed by iodine solution; C cellulase activity from strain CV2-1 revealed by Congo red; D proteinase activity from strain CV2-3 revealed by halo-casein degradation; E-AZCL-galactan hydrolyses from strain CV2-4. **Table S1.** Microbial isolates deriving from composting plant of the Experimental Station of the University of Naples Federico II at Castel Volturno (Caserta, Italy). **Table S2.** Pairwise identity matrix created by CLUSTAL of 16S rRNA gene sequences of isolated strains. The percentage of identity score between two sequences was indicated. **Table S3.** Integrated areas of chemical shift intervals for conventional ^{1}H-HRMAS spectra as normalized to the 3.49 ppm signal. **Table S4.** Integrated areas of chemical shift intervals for ^{1}H-HRMAS–NMR CPMG edited spectra as normalized to the 3.84 ppm signal. **Table S5.** Integrated areas of chemical shift intervals for ^{1}H-HRMAS–NMR DIFFUSION edited spectra as normalized to the 0.833 ppm signal.

Acknowledgements
The authors thank Vincenzo Di Meio, Responsible of Experimental Station of the University of Naples Federico II, at Castel Volturno (Caserta, Italy).

Author contributions
Conceptualization: IR, APoli and BN; methodology: LL, MK, APiccolo, APoli; formal analysis: IF, AG, AC, LL, SC, PM, APiccolo; investigation: IF, AG, AC, LL, IR, SC, PM, APiccolo; resources: APiccolo, APoli; writing—original draft preparation: all authors; writing—review and editing: all authors; supervision: APoli, BN. All authors have agreed to the published version of the manuscript. All authors read and approved the final manuscript.

Funding
This work was partially funded by Bilateral Agreement BAS/CNR Joint Project 2019–2021.

Availability of data and materials
The data presented in this study are available in the article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Institute of Biomolecular Chemistry (ICB), Consiglio Nazionale delle Ricerche (CNR), Via Campi Flegrei 34, 80078 Pozzuoli, Naples, Italy. [2]Centro Interdipartimentale di Ricerca CERMANU, Università degli Studi di Napoli Federico II, Via Università 100, 80055 Portici, Naples, Italy. [3]Dipartimento di Farmacia, Università di Salerno, 84084 Fisciano, Italy. [4]Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.

Received: 28 October 2022 Accepted: 4 January 2023
Published online: 31 January 2023

References

1. Ryckeboer J, Mergaert J, Coosemans J, Deprins K, Swings J. Microbiological aspects of biowaste during composting in a monitored compost bin. J Appl Microbiol. 2003;94:127–37. https://doi.org/10.1046/j.1365-2672.2003.01800.x.
2. Chen L, de Haro Marti M, Moore A, Falen C. Dairy compost production and use in Idaho: the composting process. University of Idaho Extension Publication. CIS 1179; 2011.
3. Liu D, Zhang R, Wu H, Xu D, Tang Z, Yu G, Xu Z, Shen Q. Changes in biochemical and microbiological parameters during the period of rapid composting of dairy manure with rice chaff. Bioresour Technol. 2011;102:9040–9. https://doi.org/10.1016/j.biortech.2011.07.052.
4. Lyon PF, Beffa T, Blanc M, Auling G, Aragno M. Isolation and characterization of highly thermophilic xylanolytic *Thermus thermophilus* strains from hot composts. Can J Microbiol. 2000;46:1029–35. https://doi.org/10.1139/w00-075.
5. Sung MH, Kim H, Bae JW, Rhee SK, Jeon CO, Kim K, Kim JJ, Hong SP, Lee SG, Yoon JH, Park YH, Baek DH. *Geobacillus toebii* sp. nov., a novel thermophilic bacterium isolated from hay compost. Int J Syst Evol Microbiol. 2002;52:2251–5. https://doi.org/10.1099/00207713-52-6-2251.
6. Han SI, Lee JC, Lee HJ, Whang KS. *Planifilum composti* sp. nov., a thermophile isolated from compost. Int J Syst Evol Microbiol. 2013;63:4557–61. https://doi.org/10.1099/ijs.0.053199-0.
7. Poli A, Laezza G, Gul-Guven R, Orlando P, Nicolaus B. *Geobacillus galactosidasius* sp. nov., a new thermophilic galactosidase-producing bacterium isolated from compost. Syst Appl Microbiol. 2011;34:419–23. https://doi.org/10.1016/j.syapm.2011.03.009.
8. Charbonneau DM, Meddeb-Mouelhi F, Boissinot M, Sirois M, Beauregard M. Identification of thermophilic bacterial strains producing thermotolerant hydrolytic enzymes from manure compost. Indian J Microbiol. 2012;52:41–7. https://doi.org/10.1007/s12088-011-0156-8.
9. Finore I, Gioiello A, Leone L, Orlando P, Romano I, Nicolaus B, Poli A. *Aeribacillus composti* sp. nov., a thermophilic bacillus isolated from olive mill pomace compost. Int J Syst Evolut Microbiol. 2017;67:4830–5. https://doi.org/10.1099/ijsem.0.002391.
10. Cabrera MÁ, Blamey JM. Biotechnological applications of archaeal enzymes from extreme environments. Biol Res. 2018;51:1–15. https://doi.org/10.1186/s40659-018-0186-3.
11. Espina G, Muñoz-Ibacache SA, Cáceres-Moreno P, Amenabar MJ, Blamey JM. From the discovery of extremozymes to an enzymatic product: roadmap based on their applications. Front Bioeng Biotechnol. 2022;9:1–9. https://doi.org/10.3389/fbioe.2021.752281.
12. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev. 2002;66:506–77. https://doi.org/10.1128/MMBR.66.3.506-577.2002.
13. Leoni C, Gattulli BAR, Pesole G, Ceci LR, Volpicella M. Amylomaltases in extremophilic microorganisms. Biomolecules. 2021;11:1–25. https://doi.org/10.3390/biom11091335.
14. Siddiqui KS. Some like it hot, some like it cold: temperature dependent biotechnological applications and improvements in extremophilic enzymes. Biotechnol Adv. 2015;33:1912–22. https://doi.org/10.1016/j.biotechadv.2015.11.001.
15. Atalah J, Cáceres-Moreno P, Espina G, Blamey JM. Thermophiles and the applications of their enzymes as new biocatalysts. Bioresour Technol. 2019;280:478–88. https://doi.org/10.1016/j.biortech.2019.02.008.
16. Ejaz U, Sohail M, Ghanemi A. Cellulases: from bioactivity to a variety of industrial applications. Biomimetics. 2021;6:1–11. https://doi.org/10.3390/biomimetics6030044.
17. Soy S, Sharma SR, Kumar NV. Bio-fabrication of thermozyme-based nanobiosensors: their components and present scenario. J Mater Sci Mater Electron. 2022;33:5523–33. https://doi.org/10.1007/s10854-022-07741-9.
18. Gupta RS, Patel S, Saini N, Chen S. Robust demarcation of 17 distinct *Bacillus* species clades, proposed as novel *Bacillaceae* genera, by phylogenomics and comparative genomic analyses: description of *Robertmurraya kyonggiensis* sp. nov. and proposal for an emended genus *Bacillus* limiting it only to the members of the Subtilis and Cereus clades of species. Int J Syst Evol Microbiol. 2020;70:6531–3. https://doi.org/10.1099/ijsem.0.004475.
19. Amore A, Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V. Industrial waste-based compost as a source of novel cellulolytic strains and enzymes. FEMS Microbiol Lett. 2013;339:93–101. https://doi.org/10.1111/1574-6968.12057.
20. López MJ, Jurado MM, López-González JA, Estrella-González MJ, Martínez-Gallardo MR, Toribio A, Suárez-Estrella F. Characterization of thermophilic lignocellulolytic microorganisms in composting. Front Microbiol. 2021;12:1–13. https://doi.org/10.3389/fmicb.2021.697480.
21. Halebian S, Harris B, Finegold SM, Rolfe RD. Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. J Clin Microbiol. 1981;13:444–8. https://doi.org/10.1128/jcm.13.3.444-448.1981.
22. Papale M, Romano I, Finore I, Lo Giudice A, Piccolo A, Cangemi S, Di Meo V, Nicolaus B, Poli A. Prokaryotic diversity of the composting thermophilic phase: the case of ground coffee compost. Microorganisms. 2021;9:1–19. https://doi.org/10.3390/microorganisms9020218.
23. Joulak I, Finore I, Nicolaus B, Leone L, Moriello AS, Attia H, Poli A, Azabou S. Evaluation of the production of exopolysaccharides by newly isolated *Halmonas* strains from Tunisian hypersaline environments. Int J Biol Macromol. 2019;138:658–66. https://doi.org/10.1016/j.ijbiomac.2019.07.128.
24. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol. 2008;74:2461–70. https://doi.org/10.1128/AEM.02272-07.
25. Finore I, Vigneron A, Vincent WF, Leone L, Di Donato P, Schiano Moriello A, Nicolaus B, Poli A. Novel psychrophiles and exopolymers from permafrost thaw lake sediments. Microorganisms. 2020;8:1–15. https://doi.org/10.3390/microorganisms8091282.
26. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1547–9. https://doi.org/10.1093/molbev/msy096.
27. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;25:4876–82. https://doi.org/10.1093/nar/25.24.4876.
28. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980;16:111–20. https://doi.org/10.1007/BF01731581.
29. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4:406–25. https://doi.org/10.1093/oxfordjournals.molbev.a040454.
30. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985;39:783–91. https://doi.org/10.2307/2408678.
31. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–9. https://doi.org/10.1093/molbev/msr121.
32. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein using the principles of protein-dye binding. Anal Biochem. 1976;72:248–54. https://doi.org/10.1006/abio.1976.9999.
33. Finore I, Poli A, Di Donato P, Lama L, Trincone A, Fagnano M, Mori M, Nicolaus B, Tramice A. The hemicellulose extract from *Cynara cardunculus*: a source of value-added biomolecules produced by xylanolytic thermozymes. Green Chem. 2016;18:2460–72. https://doi.org/10.1039/C5GC02774H.
34. Finore I, Lama L, Di Donato P, Romano I, Tramice A, Leone L, Nicolaus B, Poli A. *Parageobacillus thermantarcticus*, an antarctic cell factory: from

crop residue valorization by green chemistry to astrobiology studies. Diversity. 2019;11:1–23. https://doi.org/10.3390/d11080128.
35. Boyadzhieva I, Radchenkova N, Atanasova N, Poli A, Finore I, Kambourova M. Isolation and characterization of a thermostable pectinase by *Anoxybacillus gonensis* strain 357. C R Acad Bulg Sci. 2021;74:1757–66.
36. Wood PJ, Erfle JD, Teather RM. Use of complex formation between Congo Red and polysaccharides in detection and assay of polysaccharide hydrolases. Methods Enzymol. 1988;160:59–74. https://doi.org/10.1016/0076-6879(88)60107-8.
37. Li LL, Taghavi S, McCorkle SM, Zhang YB, Blewitt MG, Brunecky R, Adney WS, Himmel ME, Brumm P, Drinkwater C, Mead DA, Tringe SG, van der Leile D. Bioprospecting metagenomics of decaying wood: mining for new glycoside hydrolases. Biotechnol Biofuels. 2011;4:1–13. https://doi.org/10.1186/1754-6834-4-23.
38. Bailey MJ. A note on the use of dinitrosalicylic acid for determining the products of enzymatic reactions. Appl Microbiol Biotechnol. 1988;29:494–6. https://doi.org/10.1007/BF00269074.
39. Calsavara LPV, Moraes FF, Zanin GM. Thermal stability and energy of deactivation of free and immobilized cellobiase. Braz J Chem Eng. 2000;17:4–7. https://doi.org/10.1590/S0104-66322000000400047.
40. Righi V, Constantinou C, Kesarwani M, Rahme LG, Tzika AA. Effects of a small, volatile bacterial molecule on *Pseudomonas aeruginosa* bacteria using whole cell high-resolution magic angle spinning nuclear magnetic resonance spectroscopy and genomics. Int J Mol Med. 2018;42:2129–36. https://doi.org/10.3892/ijmm.2018.3760.
41. Zhang B, Halouska S, Schiaffo CE, Sadykov MR, Somerville GA, Powers R. NMR analysis of a stress response metabolic signaling network. J Proteome Res. 2011;10:3743–54.
42. Teng Q, Huang W, Collette TW, Ekman DR, Tan C. A direct cell quenching method for cell culture-based metabolomics. Metabolomics. 2009;5:199–208. https://doi.org/10.1007/s11306-008-0137-z.
43. Salaun S, Kervarec N, Potin P, Haras D, Piotto M, La Barre S. Whole-cell spectroscopy is a convenient tool to assist molecular identification of cultivatable marine bacteria and to investigate their adaptive metabolism. Talanta. 2010;80:1758–70. https://doi.org/10.1016/j.talanta.2009.10.020.
44. Kalpana S, Sahoo RK, Gaur M, Subudhi E. Cellulolytic thermophilic microorganisms in white biotechnology: a review. Folia Microbiol. 2020;65:25–43. https://doi.org/10.1007/s12223-019-00710-6.
45. Kamm B, Kamm M. Principles of biorefineries. Appl Microbiol Biotech. 2004;64:137–45. https://doi.org/10.1007/s00253-003-1537-7.
46. Kasavi C, Finore I, Lama L, Nicolaus B, Oliver SG, Toksoy OE, Kirdara B. Evaluation of industrial *Saccharomyces cerevisiae* strains for ethanol production from biomass. Biomass Bioenergy. 2012;45:230–8. https://doi.org/10.1016/j.biombioe.2012.06.013.
47. Lama L, Tramice A, Finore I, Anzelmo G, Calandrelli V, Pagnotta E, Tommonaro G, Poli A, Di Donato P, Nicolaus B, Fagnano M, Mori M, Impagliazzo A, Trincone A. Degradative actions of microbial xylanolytic activities on hemicelluloses from rhizome of *Arundo donax*. AMB Express. 2014;4:1–9. https://doi.org/10.1186/s13568-014-0055-6.
48. Awasthi MK, Tarafdar A, Gaur VK, Amulya K, Narisetty V, Yadav DK, Sindhu R, Bino P, Negi T, Pandey A, Zhang Z, Sirohi R. Emerging trends of microbial technology for the production of oligosaccharides from biowaste and their potential application as prebiotic. Int J Food Microbiol. 2022;368: 109610. https://doi.org/10.1016/j.ijfoodmicro.2022.109610.
49. Cimini D, Argenzio O, D'Ambrosio S, Lama L, Finore I, Finamore R, Pepe O, Faraco V, Schiraldi C. Production of succinic acid from *Basfia succiniciproducens* up to the pilot scale from *Arundo donax* hydrolysate. Bioresour Technol. 2016;222:355–60. https://doi.org/10.1016/j.biortech.2016.10.004.
50. Harindintwali JD, Zhou J, Yu X. Lignocellulosic crop residue composting by cellulolytic nitrogen-fixing bacteria: a novel tool for environmental sustainability. Sci Total Environ. 2020;715:1–13. https://doi.org/10.1016/j.scitotenv.2020.136912.
51. Kumar V, Sangwan P, Singh D, Gill PK. Global scenario of industrial enzyme market. In: Beniwal V, Sharma AK, editors. Industrial enzymes: trends, scope and relevance. 1st ed. New York: Nova Science Pub Inc; 2014. p. 173–96.
52. Bakare MK, Adewale IO, Shonukan OO. Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens*. Afr J Biotechnol. 2005;4:898–904.
53. Liang YL, Zhang Z, Wu M, Wu Y, Feng JX. Isolation, screening, and identification of cellulolytic bacteria from natural reserves in the subtropical region of China and optimization of cellulase production by *Paenibacillus terrae* ME27-1. BioMed Res Int. 2014;2014:1–13. https://doi.org/10.1155/2014/512497.
54. Mingardon F, Bagert JD, Maisonnier C, Trudeau DL, Arnold FH. Comparison of family 9 cellulases from mesophilic and thermophilic bacteria. Appl Environ Microbiol. 2011;77:1436–42. https://doi.org/10.1128/AEM.01802-10.
55. Sang-Mok L, Koo YM. Pilot-scale production of cellulase using *Trichoderma reesei* Rut C-30 in fed-batch mode. J Microbiol Biotechnol. 2001;11:229–33. https://doi.org/10.1007/s12257-007-0149-y.
56. Kuhad RC, Gupta R, Singh A. Microbial cellulases and their industrial applications. Enzyme Res. 2011;2011:1–10. https://doi.org/10.4061/2011/280696.
57. Kumar S, Srivastava N, Sen Gupta B, Kuhad RC, Gomes J. Lovastatin production by *Aspergillus terreus* using lignocellulose biomass in large scale packed bed reactor. Food Bioprod Process. 2014;92:416–24. https://doi.org/10.1016/j.fbp.2013.10.007.
58. Sukumaran RK, Singhania RR, Pandey A. Microbial cellulases—production, applications, and challenges. J Sci Ind Res. 2005;64:832–44.
59. Seo JK, Park TS, Kwon IH, Piao MY, Lee CH, Ha JK. Characterization of cellulolytic and xylanolytic enzymes of *Bacillus licheniformis* JK7 isolated from the rumen of a native Korean goat. Asian-Australas J Anim Sci. 2013;26:50–8. https://doi.org/10.5713/ajas.2012.12506.
60. Yang G, Yang D, Wang X, Cao W. A novel thermostable cellulase-producing *Bacillus licheniformis* A5 acts synergistically with *Bacillus subtilis* B2 to improve degradation of Chinese distillers' grains. Bioresour Technol. 2021;325:1–9. https://doi.org/10.1016/j.biortech.2021.124729.
61. de Marco VG, Heck K, Martos ET, van der Sand ST. Purification and characterization of a thermostable alkaline cellulase produced by *Bacillus licheniformis* 380 isolated from compost. An Acad Bras Cienc. 2017;89:2359–70. https://doi.org/10.1590/0001-3765201720170408.
62. Nallusamy S, Amira AZ, Saif AB, Abdulkhadir E, Elsadig AE. Isolation and characterization of cellulolytic *Bacillus licheniformis* from compost. Afr J Biotechnol. 2016;15:2434–46. https://doi.org/10.5897/ajb2016.15641.
63. Kazeem MO, Shah UKM, Baharuddin AS, Abdul Rahman NA. Enhanced cellulase production by a novel thermophilic *Bacillus licheniformis* 2D55: characterization and application in lignocellulosic saccharification. BioResources. 2016;11:5404–23. https://doi.org/10.15376/biores.11.2.5404-5423.
64. Rastogi G, Bhalla A, Adhikari A, Bischoff KM, Hughes SR, Christopher LP, Sani RK. Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. Bioresour Technol. 2010;101:8798–806. https://doi.org/10.1016/j.biortech.2010.06.001.
65. De Corato U. Agricultural waste recycling in horticultural intensive farming systems by on-farm composting and compost-based tea application improves soil quality and plant health: a review under the perspective of a circular economy. Sci Total Environ. 2020;738:1–22. https://doi.org/10.1016/j.scitotenv.
66. Ajeje SB, Hu Y, Song G, Peter SB, Afful RG, Sun F, Asadollahi MA, Amiri H, Abdulkhani A, Sun H. Thermostable cellulases/xylanases from thermophilic and hyperthermophilic microorganisms: current perspective. Front Bioeng Biotechnol. 2021;9:1–19. https://doi.org/10.3389/fbioe.2021.794304.
67. Tashiro Y, Matsumoto H, Miyamoto H, Okugawa Y, Pramod P, Miyamoto H, Sakai K. A novel production process for optically pure L-lactic acid from kitchen refuse using a bacterial consortium at high temperatures. Bioresour Technol. 2013;146:672–81. https://doi.org/10.1016/j.biortech.2013.07.102.
68. Yue S, Mizoguchi T, Kohara T, Zhang M, Watanabe K, Miyamoto H, Tashiro Y, Sakai K. Meta-fermentation system with a mixed culture for the production of optically pure L-lactic acid can be reconstructed using the minimum isolates with a simplified pH control strategy. Biotechnol J. 2021;16:1–11. https://doi.org/10.1002/biot.202100277.
69. Inabu Y, Taguchi Y, Miyamoto H, Etoh T, Shiotsuka Y, Fujino R, Okada T, Udagawa M, Tsuji N, Matsuura M, Tsuboi A, Kato T, Kodama H, Ohno H, Takahashi H. Development of a novel feeding method for Japanese black

calves with thermophile probiotics at postweaning. J Appl Microbiol. 2022;132:3870–82. https://doi.org/10.1111/jam.15519.

70. Miyamoto H, Seta M, Horiuchi S, Iwasawa Y, Naito T, Nishida A, Miyamoto H, Matsushita T, Itoh K, Kodama H. Potential probiotic thermophiles isolated from mice after compost ingestion. J Appl Microbiol. 2013;114:1147–57. https://doi.org/10.1111/jam.12131.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.