Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Cellulose degrading bacteria isolated from industrial samples and the gut of native insects from Northwest of Argentina

Adriana P. Manfredi[1,2], Nora I. Perotti[1,2] and María A. Martínez[1,2]

[1] PROIMI Planta Piloto de Procesos Industriales Microbiológicos, CONICET, Tucumán, Argentina
[2] Facultad de Ciencias Exactas y Tecnología, Universidad Nacional de Tucumán, Tucumán, Argentina

The raw materials used to produce bioethanol mostly are food crops, which has led to conflicts on food security. It is, therefore, recommended the gradual replacement for second generation substrates such as lignocellulosic materials. Herein, cellulolytic bacteria were isolated from the gut content of native larvae from *Lepidoptera*, *Coleoptera*, and adults of *Isoptera*. Few environmental samples from the pulp and paper feedstock were also assessed. A total of 233 isolates were obtained using enrichment cultures and classic criteria. Interestingly, several halo-forming colonies were found to be bacterial consortia that presented difficulties to take apart the microbial members. Those pure isolates which hydrolyzed cellulose in larger extend (45 strains) were selected and identified by means of 16S rRNA sequence analysis. *Firmicutes* was the prevalent phylum (62.2%) being *Bacillus* spp. the most frequent genus, while *Paenibacillus*, *Brevibacillus*, *Cohnella*, and *Staphylococcus* species were less frequent. The phylum *Actinobacteria* (6.7%) was represented by isolates related to *Agromyces* spp. and *Microbacterium* spp. Regarding Gram-negative bacteria (31.1%), the more depicted genus was *Pseudomonas* spp., and members of *Achromobacter* spp., *Enterobacter* spp., and *Bacteroidetes* phylum were also selected. These native bacterial strains are expected to enlarge the cellulolytic toolbox for efficient biomass deconstruction.

Keywords: Biomass degradation / Cellulolytic bacteria / Enrichment cultures / Industrial samples / Xylophagous insects

Received: April 29, 2015; accepted: August 2, 2015

DOI 10.1002/jobm.201500269

Introduction

Enzymatic hydrolysis of lignocellulosic material is a major bottleneck toward the development of industrial feasible processes for bioethanol production from resources that do not compete with food crops [1]. Our understanding of the degradation of lignocellulosic material is still limited because of the vast diversity of organisms present in most cellulose degrading environments, the diverse strategies that display in order to deconstruct vegetal biomass and the inability to culture most of them [2, 3]. Thus, the description and characterization of new microorganisms and/or enzymes able to degrade efficiently the plant biomass is critical to contribute to the development of viable processes and to deepen the understanding of such processes.

Microbial species able to achieve the deconstruction of plant cell wall produce an extensive repertoire of enzymes targeting the numerous linkages present in this composite structure. This is commonly produced by multiple microorganisms with complementary capabilities [2, 4]. These features have been assessed by using various approaches, such as the selection of microbial consortia able to efficiently degrade plant biomass [5, 6]; by the development of recombinant organisms [1, 7]; or by means of massive sequencing projects [8, 9]. In this context, Bacteria are being extensively considered as a source of novel cellulases due to their higher growth rate and their extensive repertoire of glycoside hydrolases [10–12]. Despite the inability to culture most of the microorganisms present in cellulose degrading environments, the isolation of pure strains allow to a better understanding of the metabolic behavior of microorganisms of interest.

Correspondence: Dr. M. Alejandra Martínez, PROIMI Planta Piloto de Procesos Industriales Microbiológicos, CONICET, Tucumán 4000, Argentina
E-mail: ale.martinez.unt@gmail.com
Phone: +54-381-4344888
Fax: +54-381-4344887

With the aim to isolate a variety of efficient cellulolytic bacteria, over 200 pure isolates were recovered from gut samples of locally collected insects and from sugarcane associated facilities.

Materials and methods

Samples used for bacteria isolation

Gut samples. They were obtained from phytophagous and xylophagous insects. The guts were dissected from larvae of *Lepidoptera – Spodoptera frugiperda* and *Diatraea saccharalis – Coleoptera* (wood-feeding beetle); and adult termites (*Isoptera*). *S. frugiperda* are leaf eaters and constitutes a corn plague in Argentina and were kindly provided by Dr. Virla. *D. saccharalis*, also known as sugarcane borer, is responsible of economic damage in sugar cane fields in Northwest of Argentina. Larvae of wood-feeding beetle and termites were also collected from regional plagued wood.

Adult insects and larvae collected were externally sterilized with 70% ethanol and dissected. Up to eight individual gut of each sample were pooled and crushed into a 50-ml screw-cap disposable tube containing 10 ml of 0.9% (w/v) sterile sodium chloride (saline solution).

Environmental samples. Samples of biological liquor from bagasse processing were collected from a paper mill in Tucumán, Argentina (26°56′47″S 65°22′16″W). The treatment of bagasse fibers is performed in compacted sugarcane bagasse piles of 45–50 m high and a base of approximately 150–200. Samples of recirculating liquor were taken from the draining channels at several points at the base of the piles.

Culture media

The culture media used were (i) DTSB, contained g/l: soy tryptone 8.5, glucose 1.25, NaCl 2.5, and K_2HPO_4 1.25; (ii) DNB containing in g/l: beef extract 1.5 and peptone 2.5; (iii) modified VL70 – mVL70 – was used [13]. It was composed in g/l: 3-[N-morpholino] propanesulfonic acid 2.09, $MgSO_4$ 0.024, $CaCl_2$ 0.035, $(NH4)_2HPO_4$ 0.052, KH_2PO_4 0.052, and yeast extract 0.1. The pH was adjusted to 7.0 with a solution of 200 mM NaOH and 100 mM KOH. Carboxymethyl cellulose (CMC low viscosity, Sigma), milled bagasse, Avicel cellulose (Sigma–Aldrich) were the carbon sources added at a final concentration of 10 g/l. Sugarcane bagasse (SCB) was collected from a local sugar–alcohol mill (Tucumán, Argentina), air-dried and milled to pass through a 20 mesh sieve. Also, NaOH-pretreated SCB was prepared using a 2% (w/v) NaOH solution and a solid loading of 10% and treated into an autoclave at 121 °C during 1 h. The solid fraction was filtered (500 mesh), and washed with hot water (*c.a.* 90 °C) until pH 6–7. All the solids were oven-dried at 85 °C and stored.

Enrichment and isolation of cellulose-degrading bacteria

One milliliter of each well-blended gut sample or 1 ml of draining liquor of bagasse processing were inoculated within 12 h of collection into 125 ml flasks containing 20 ml culture media and incubated with orbital agitation (150 rpm) at 30 °C. All flasks contained a filter paper strip (Whatman N° 1) of 1 × 2 cm. Sterile controls medium were incubated. Growths from each flask were transferred weekly to fresh medium during 6 weeks (seed volume of 10%, v/v). Aliquots (100 μl) from 2, 4, and 6 weeks-old enrichment cultures were processed for isolation. The samples were serially diluted (10^{-1}–10^{-6}) using sterilized saline solution 0.9% (w/v) NaCl and aliquots of 100 μl were streaked onto cellulose Congo-Red agar [14] (containing g/l: KH_2PO_4 0.5, $MgSO_4$ 0.25, carboxymethyl cellulose 2, agar 15, Congo-Red 0.2, gelatin 2; pH 6.8–7.2) and incubated at 30 °C. To ensure isolation, single colonies were picked, re-streaked onto solidified DTSB (agar 1.6%, w/v) and carefully checked for homogeneous morphology using 40× magnification (Olympus CX31 optical microscope). The colonies were evaluated to ensure purity by means of the observation of texture, color, brightness, margins, and convexity.

Enzyme assays

Semi-quantitative assessment was performed over colonies showing significant hydrolysis halos according to the ratio between diameter of the halo plus the diameter of the colony and the diameter of de colony.

Filter paper enzymatic activity (FPase) and endoglucanase (β 1–4 endoglucanase, E.C. 3.2.1.4) activity were determined by measuring the amount of reducing sugars released from filter paper and amorphous cellulose, respectively, using the dinitrosalicylic (DNS) method [15] as is described below. To evaluate endoglucanase activity, isolates were grown in 20 ml of DTSB amended with 10 g/l CMC. Incubation was performed at 30 °C and 150 rpm. After 2–5 days of incubation period, which varied for the different isolates, cultures were centrifuged (10,000×*g*, 10 min, 4 °C) and the extracellular crude extract was used as enzyme source for activity measurements and zymographic analysis. Cellular pellets were kept at –20 °C for DNA extraction. Endoglucanase was assayed incubating 0.05 ml of extracellular crude extracts with 0.45 ml of 100 mM sodium phosphate buffer (pH 6.5) containing 2% (w/v) CMC low viscosity (Sigma). After 30 min of incubation at

50 °C, reactions were stopped by adding 0.50 ml of 3, 5-dinitrosalicylic acid reagent [14]. Identical procedure was followed for the determination of xylanolytic activity (using 1% (w/v) birchwood xylan-Sigma as substrate).

Qualitative filter paper activity of all isolates was evaluated as total cellulolytic potential in 100 ml flasks containing 20 ml of mVL70 medium including a Whatman N° 1 filter paper strip (1 × 2 cm). The cultures were incubated at 30 °C and 150 rpm for a maximum of 7 days and checked daily for evidence of filter paper degradation. The strains capable of degrading filter paper were selected for further quantitative analysis. This was performed incubating 0.05 ml of enzymatic crude extract with a strip of Whatman N° 1 FP (1 × 6 cm) in 0.45 ml of sodium phosphate buffer (pH6.5). The assays were carried out at 50 °C for 60 min. Reducing sugars released were determined with the DNS reagent as described above.

One international unit (IU) of enzymatic activity (FPase, CMCase, and xylanase) was defined as the amount of enzyme that releases 1 μmol of reducing sugars (measured as glucose) per milliliter per minute under the described conditions. All samples were analyzed in triplicate and mean values and standard deviation were calculated.

For zymographic analysis, extracellular crude enzyme samples were loaded on 12% non-denaturing polyacrylamide gels (ND-PAGE). After electrophoresis, gels were incubated at 30 °C for 30 min in substrate buffers of 100 mM sodium phosphate buffer (pH 6.5), containing 1% (w/v) CMC, and stained with 0.1% (w/v) Congo Red solution. After at least two washes with 1 M NaCl during 15 min were performed until visualization of clear bands of enzyme activity, apparent molecular weight was estimated using an adequate marker (HMW-GE Healthcare, 669–66 kDa), which was revealed by the silver staining method.

DNA isolation and 16S rRNA gene amplification and analysis

The Cetyl trimethylammonium bromide (CTAB) method [16] was used for the genomic DNA isolation of selected strains from the reserved cellular pellets (Enzyme assays). Genomic samples were used to amplify the 16S rRNA genes using universal primers F27, and R1492 [17]. Also, R907 (5′CCGTCAATTCCTTTGAGTTT3′) and R518 (5′ATTACCGCGGCTGCTGG3′) were used. The reaction mixture, on a final volume of 25 μl, contained 5 μl of 5× polymerase buffer, 0.5 μM of each primer, 200 μM of dNTPs, and 1 U of Go-Taq DNA polymerase (Promega, USA). The reaction was run on a Mastercycler Gradient (Eppendorf, Germany), using the following cycle: 94 °C 4 min, 30 cycles of 45 s at 94 °C, 45 s at 55 °C, and 90 s at 72°C, with a final extension of 7 min at 72 °C. Amplification products were then checked to confirm size and quality by submerged electrophoresis in 0.8% (w/v) agarose gels stained with Gel RedTM (Biotium).

Sequences were obtained by direct sequencing from Macrogen facilities (Macrogen, Korea). The reads obtained were edited and individually inputted online into the EZ taxon server [18] and through the nucleotide blast tool (http://blast.ncbi.nlm.nih.gov/) to identify the most related genera of the isolates. All sequences were recorded in public databases under the accession numbers FJ976043.1 (GenBank), LN829557–LN829597, and LN832405–LN832407 (European Nucleotide Archive).

Results

Enrichment and isolation of cellulolytic bacteria

In order to augment the framework of cellulose-degrading bacteria, an enrichment-isolation procedure from the samples was performed. Samples were inoculated into different media – DNB and DTSB, and mVL70 – within the 12 h of collection to increase bacterial recovery [13, 19]. Enrichment cultures at pH values around neutrality (pH 7.0 ± 1) were incubated aerobically at 30 °C since aerobic and facultative anaerobic microbes also occur in insect guts [20]. As a result, bacterial growth ($OD_{600\,nm} > 0.9$) was observed in diluted nutritive media amended with SCB, Avicel, and CMC. On the contrary, cultures into the mineral based medium mVL70 + SCB showed poor growth ($OD_{600\,nm} < 0.5$). Aliquots of enrichment cultures were harvested at intervals of 2 weeks and streaked onto cellulose plates containing Congo Red. This dye stains the agar media containing CMC and produces clear areas where this substrate was degraded, providing a rapid and sensitive screening test to evaluate the cellulolytic potential of bacteria. After 72–96 h of incubation at 30 °C, colonies showing significant hydrolysis halos (ratio between diameter of the halo plus the diameter of the colony and the diameter of de colony ≥2.5 mm) were isolated (Fig. 1). The colony morphology of each isolate was carefully checked to ensure the recovery of pure cultures. It was noticeable that several colonies producing hydrolysis halo contained bacterial consortia instead of single strains. Thus, efforts to retrieve pure cultures required several sub-cultivations of single colonies and as a result, a total of 233 pure isolates were obtained.

The isolated cellulolytic strains were mainly recovered from microbial enrichments developed in mVL70, which presented scarce bacterial development as was mentioned. It was noticeable that mVL70 with SCB as carbon

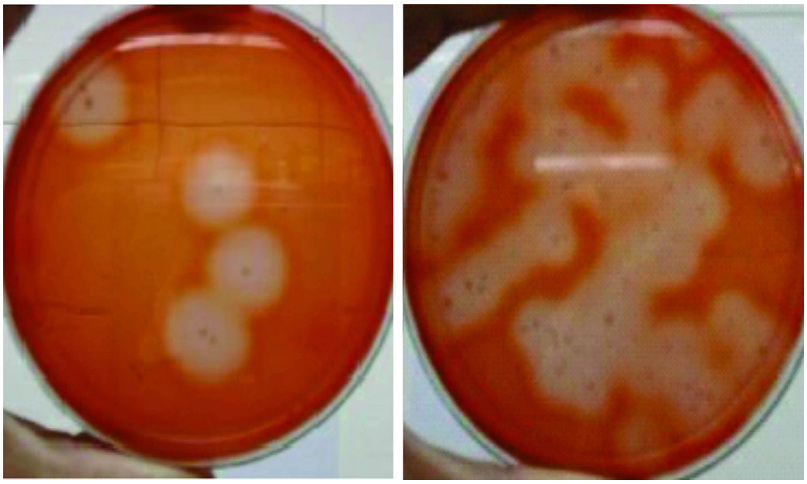

Figure 1. Zone of clearance on cellulose obtained after 48 h of incubation onto Congo Red agar plates from enrichments cultures. The formation of clearing zone around the colonies was indicative of the production of extracellular cellulases.

source produced 42.1% of all cellulolytic isolates, while mVL70 amended with CMC produced only 6.4% of them (Fig. 2A).

Screening of cellulolytic activity in culture supernatants

The growth observed of most of the isolates into mineral based media was frequently poor, reaching $OD_{600\,nm} < 0.5$. Hence, all isolates were grown in DTSB amended with CMC 10 g l^{-1} to maximize biomass growth for enzyme activity detection and incubated during 2–5 days. Most of the 233 isolates displayed poor extracellular β-1,4-endoglucanase activity. As is shown in Fig. 2B, 13.3% of all strains displayed enzymatic activities (EA) ranging from 0.10 to 1.30 IU ml^{-1}. Mild evidence of filter paper (FP) degradation was observed in 4.3% of the total isolates, whereas only 1.7% showed both, enzyme activity with FP as substrate (>0.1 IU ml^{-1}) and clear visual evidence of FP degradation. It was unexpected that FPase activity was not quantifiable by means of the DNS method, although similar observations were previously reported [21]. Moreover, it was striking to observe that some of our isolates do not exhibit this capability permanently, yet some of them (e.g., *Bacillus* sp. AR03 and *Bacillus* sp. AR491) were able to produce extensive clarification of NaOH-pretreated SCB (Pisa H., personal comm.). These observations will require further assays in order to be appropriately explained.

As a result, 45 pure isolates were selected for further characterization on the basis of different colony morphology, evidence of FP degradation, which is reported here qualitatively, and/or endoglucanase activity ≥0.10 IU ml^{-1} (Table 1).

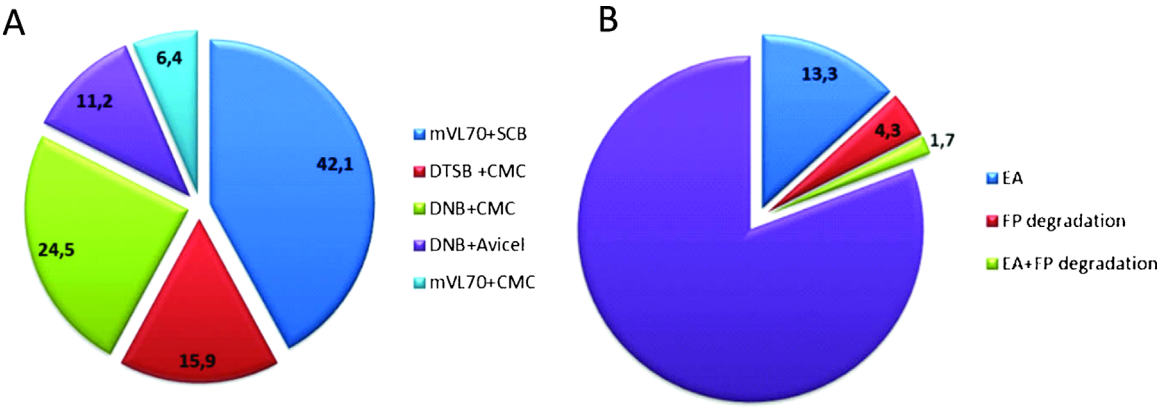

Figure 2. Distribution of the 233 cellulose degrading pure cultures isolates obtained (A) according to the different enrichment media utilized and (B) conforming to the cellulolytic activity evaluated. EA, enzymatic activity; FP, filter paper.

Table 1. Taxonomic, cellulolytic activity, and source description of the 45 selected bacterial isolates.

Isolate	Closest neighbor	Accession number	EA	FP	EA + FP	EA [IU ml^{-1}]	Source of isolation	Isolation medium
AR03	*Bacillus tequilensis* KCTC 13622(T)	LN829557			x	1.30 ± 0.15	Biological liquor	DNB + CMC
AR17	*Bacillus subtilis subsp. Subtilis* NCIB 3610(T)	LN829558	x			0.16 ± 0.03	Biological liquor	DNB + CMC
AR40-1	*Bacillus anthracis* ATCC 14578(T)	LN829559	x			0.22 ± 0.03	Biological liquor	DNB + CMC
AR98	*Bacillus sonorensis* NBRC 101234(T)	LN829560	x			0.46 ± 0.05	Biological liquor	DNB + CMC
AR138	*Bacillus tequilensis* KCTC 13622(T)	LN829561	x			0.18 ± 0.03	Biological liquor	DNB + CMC
AR141	*Bacillus cereus* ATCC 14579(T)	LN829562	x			0.17 ± 0.03	Biological liquor	DNB + CMC
AR349-1	*Bacillus firmus* NCIMB 9366(T)	LN829563	x			0.34 ± 0.04	*S. frugiperda* larvae	DNB + Avicel
AR349-2	*Bacillus sonorensis* NBRC 101234(T)	LN829564	x			0.29 ± 0.04	*S. frugiperda* larvae	DNB + Avicel
AR352	*Bacillus tequilensis* KCTC 13622(T)	LN829565	x			0.42 ± 0.05	*S. frugiperda* larvae	DNB + Avicel
AR384	*Bacillus siamensis* KCTC 13613(T)	LN829566	x			0.44 ± 0.05	*D. saccharalis* larvae	DNB + CMC
AR391	*Bacillus cereus* ATCC 14579(T)	LN829567	x			0.27 ± 0.02	*D. saccharalis* larvae	VL70 + SCB
AR398	*Bacillus amyloliquefaciens subsp. plantarum* FZB42(T)	LN829568	x			0.43 ± 0.05	*D. saccharalis* larvae	DNB + CMC
AR401	*Bacillus safensis* FO-36b(T)	LN829569	x			0.50 ± 0.05	*D. saccharalis* larvae	VL70 + CMC
AR408	*Bacillus tequilensis* KCTC 13622(T)	LN829570	x			0.80 ± 0.12	*D. saccharalis* larvae	VL70 + SCB
AR409	*Bacillus safensis* FO-36b(T)	LN829571		x		–	*D. saccharalis* larvae	VL70 + SCB
AR426	*Bacillus anthracis* ATCC 14578(T)	LN829572	x			0.36 ± 0.04	*Coleoptera* larvae	VL70 + SCB
AR434-1	*Bacillus anthracis* ATCC 14578(T)	LN829573		x		–	*Coleoptera* larvae	VL70 + SCB
AR463	*Bacillus siamensis* KCTC 13613(T)	LN829574	x			0.20 ± 0.03	*Coleoptera* larvae	VL70 + SCB
AR491	*Bacillus siamensis* KCTC 13613(T)	LN829575	x			0.32 ± 0.04	*Isoptera*	VL70 + SCB
AR493	*Bacillus amyloliquefaciens subsp. plantarum* FZB42(T)	LN829576	x			0.38 ± 0.04	*Isoptera*	VL70 + SCB
AR247	*Paenibacillus cineris* LMG 18439(T)	LN829577	x			0.14 ± 0.02	Biological liquor	DNB + CMC
AR460-1	*Paenibacillus lautus* NRRL NRS-666(T)	LN829578	x			0.25 ± 0.03	*D. saccharalis* larvae	DTSB + CMC
AR485	*Paenibacillus cineris* LMG 18439(T)	LN829579			x	0.15 ± 0.02	*Isoptera*	VL70 + SCB
AR489	*Paenibacillus cineris* LMG 18439(T)	LN829580				0.18 ± 0.02	*Isoptera*	VL70 + SCB
AR92	*Cohnella formosensis* CC-Alfalfa-35(T)	FJ976043.1	x			0.15 ± 0.03	Biological liquor	DNB + CMC
AR460-2	*Brevibacillus parabrevis* IFO 12334(T)	LN829581	x			0.12 ± 0.03	*D. saccharalis* larvae	DTSB + CMC
AR230	*Staphylococcus warneri* ATCC 27836(T)	LN829582	x			0.10 ± 0.03	Biological liquor	DNB + CMC
AR362	*Staphylococcus warneri* ATCC 27836(T)	LN829583	x			0.15 ± 0.03	*S. frugiperda* larvae	DNB + Avicel
AR18	*Microbacterium paraoxydans* CF36(T)	LN832405		x		–	Biological liquor	DNB + CMC
AR462-2	*Microbacterium paraoxydans* CF36(T)	LN829584		x		–	*Coleoptera* larvae	VL70 + SCB
AR488	*Agromyces mediolanus* DSM 20152(T)	LN829585		x		–	*Isoptera*	VL70 + SCB
AR422	*Pseudomonas aeruginosa* JCM 5962(T)	LN832407	x			0.11 ± 0.03	*Coleoptera* larvae	VL70 + SCB
AR429	*Pseudomonas aeruginosa* JCM 5962(T)	LN829586	x			0.38 ± 0.05	*Coleoptera* larvae	VL70 + SCB
AR447	*Pseudomonas hibiscicola* ATCC 19867(T)	LN829587		x		–	*Coleoptera* larvae	VL70 + SCB
AR462-1	*Pseudomonas stutzeri* ATCC 17588(T)	LN829588		x		–	*Coleoptera* larvae	VL70 + SCB
AR470	*Pseudomonas plecoglossicida* FPC951(T)	LN829589		x		–	*Coleoptera* larvae	VL70 + SCB
AR471	*Pseudomonas guguanensis* CC-G9A(T)	LN829590		x		–	*Coleoptera* larvae	VL70 + SCB
AR490	*Pseudomonas aeruginosa* JCM 5962(T)	LN829591	x			0.37 ± 0.04	*Isoptera*	VL70 + SCB
AR492-2	*Pseudomonas aeruginosa* JCM 5962(T)	LN829592			x	0.30 ± 0.04	*Isoptera*	VL70 + SCB
AR451	*Enterobacter cloacae subsp. dissolvens* LMG 2683(T)	LN829595		x		–	Coleoptera larvae	VL70 + SCB
AR476-1	*Achromobacter insolitus* LMG 6003(T)	LN829593	x			0.12 ± 0.03	*D. saccharalis* larvae	DTSB + CMC
AR476-2	*Achromobacter insolitus* LMG 6003(T)	LN829594	x			0.23 ± 0.06	*D. saccharalis* larvae	DTSB + CMC
AR11	*Chryseobacterium contaminans* C26(T)	LN829596	x			0.13 ± 0.03	Biological liquor	DNB + CMC
AR13	*Chryseobacterium contaminans* C26(T)	LN832406	x			0.17 ± 0.02	Biological liquor	DNB + CMC
AR126	*Sphingobacterium multivorum* IAM14316(T)	LN829597	x			0.10 ± 0.03	Biological liquor	DNB + CMC

EA, enzymatic activity; FP, filter paper degradation.

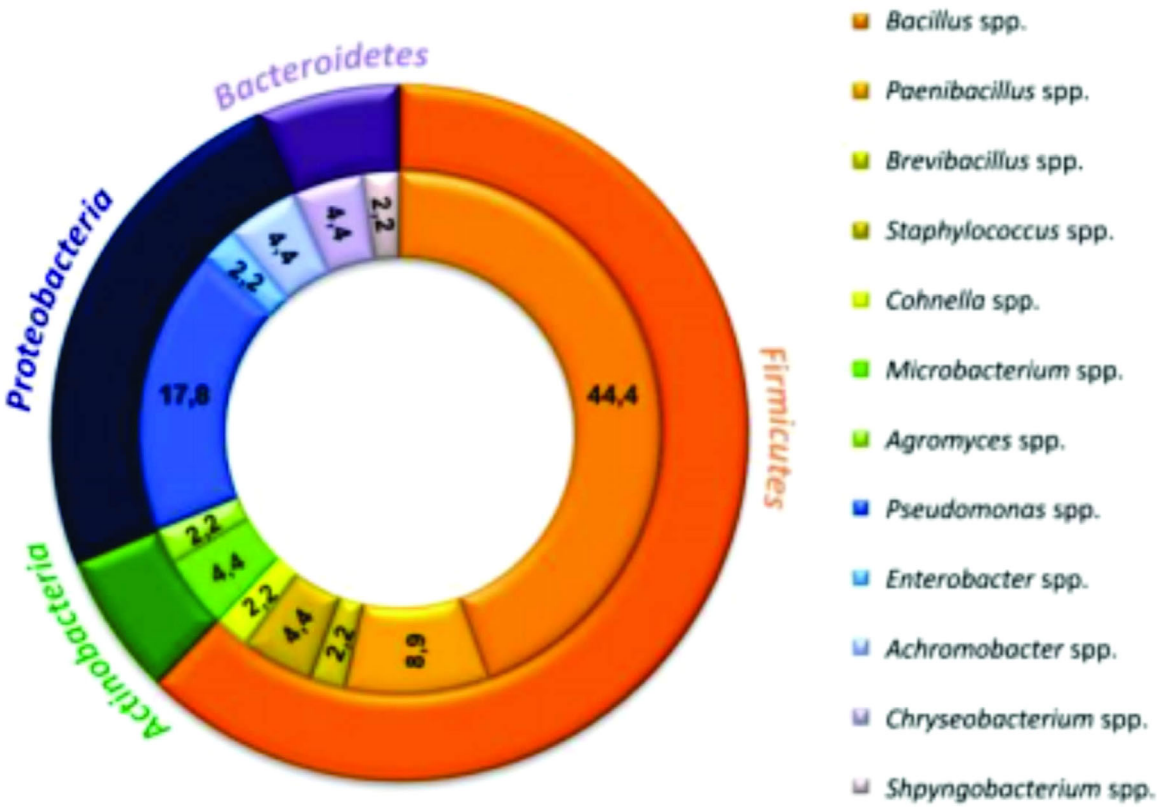

Figure 3. Taxonomic distribution of the 45 isolates selected on the basis of their cellulolytic potential.

Description of pure isolates

Firmicutes. Most of the 45 selected isolates were rods of different lengths, frequently showing endospore formation and motility. According to the taxonomic identification on the basis of 16S rRNA sequence, *Firmicutes* was the prevalent phylum observed representing 62.2% of all selected bacteria and comprising *Bacillus* spp. and *Paenibacillus* spp. as the most frequent genera (Fig. 3). It is worth to mention that several *Bacillus* spp. isolates of the subtilis group were not distinguishable by 16S rRNA sequence, fact that may reflect their numerical abundance. Nevertheless, these isolates were individually analyzed since they presented different behavior, source, and colony morphology. As an example, *Bacillus* spp. isolates AR03 and AR408 from industrial liquor and from the gut of native larvae, respectively, were almost identical in terms of their 16S rRNA sequence; both strains showed the highest CMCase activity values but only *Bacillus* sp. AR03 was able to produce some extent of FP degradation after 48 h of cultivation in DTSB (Table 1). It was observed that several *Bacillus* spp. isolates evaluated were not able to efficiently grow ($OD_{600\,nm} < 0.2$), nor produce significant CMCase activity in mineral-based media. Thus, optimization of the media culture composition and the growth conditions for the isolates must be further done in order to improve the enzyme production.

The *Paenibacillaceae* family (13.3%) was represented by members of *Paenibacillus*, *Brevibacillus*, and *Cohnella* genera as is depicted in Fig. 3. Besides their cellulolytic potential, it was observed as important xylanolytic activity in all selected *Paenibacillaceae* isolates. For instance, *Paenibacillus* sp. AR247 and *Cohnella* sp. AR92 produced $5.3 \pm 0.15\,IU\,ml^{-1}$ and $15.1 \pm 0.21\,IU\,ml^{-1}$ of endo-β-1,4-endoxylanase activity after 96 and 120 h of cultivation, respectively. The CMCase activity of the isolates of this group presented values below $0.3\,IU\,ml^{-1}$ when the culture broth evaluated was obtained from DTSB-CMC medium (Table 1). It was remarkable to observe that the zymographic analysis of *Cohnella* sp. AR92 revealed at least four bands were observed when it was cultivated in DTSB supplemented with NaOH-pretreated SCB (Fig. 4). Other Gram-positive, non-motile cocci isolates, designated as AR230 and AR362, were taxonomically clustered with *Staphylococcus warneri* regardless of their different origin (Table 1).

Other Gram-positive bacteria. Two isolates were assigned to the *Microbacterium* genus: AR18 and AR462-

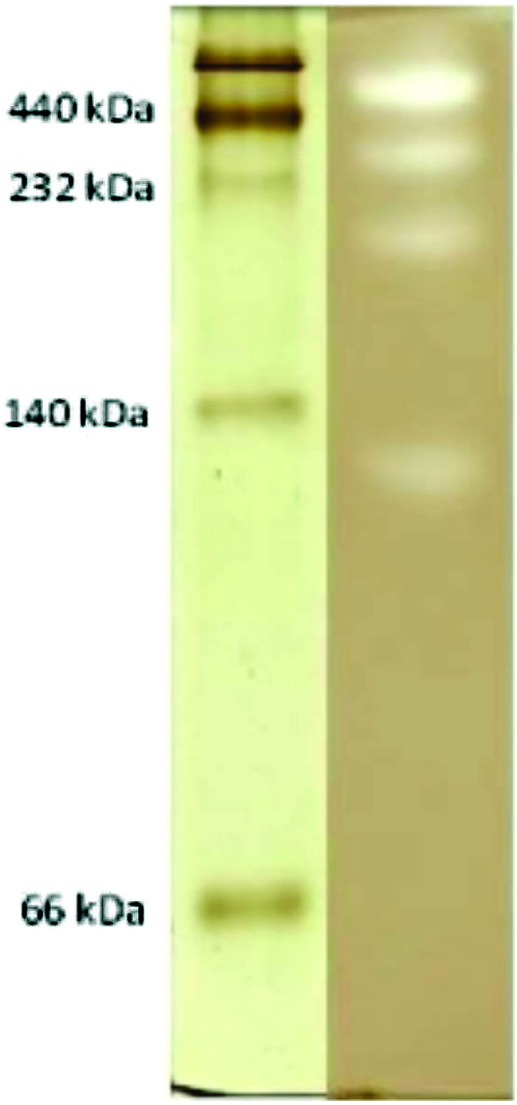

Figure 4. Zymographic profile of *Cohnella* sp. AR92 developed on ND-PAGE 12%. Lane 1, Molecular weight marker. Lane 2, CMCase activity, showing four bands with apparent molecular masses of 500, 306, 206, and 122 kDa.

2, which were retrieved from enrichment cultures inoculated with industrial liquor and with gut of wood-feeding beetle larvae samples, respectively. Also, the isolate AR488 recovered from termite guts was related to *Agromyces* spp. (Table 1).

In spite of the fact that the *Actinobacteria* isolated here showed low CMCase activity (ca. 0.02 IU ml^{-1}) and mild FP degradation, preliminary results regarding *Microbacterium* spp. evidenced xylanolytic activity when were grown in mineral-based culture media containing lactose (data not shown).

Proteobacteria and Bacteroidetes. Pseudomonas spp. is the second most frequent genera isolated in this work, representing 17.8% of all selected bacteria (Fig. 3). Several *Pseudomonas* spp. isolates exhibited mild FP degradation. As was mentioned before, this was a not constant behavior and was frequently lost after subcultures of some of the evaluated strains. However, this property was considered here as evidence of the cellulolytic potential of *Pseudomonas* spp. and other isolates described here (Table 1).

Besides the fact that most of the members of the phylum *Proteobacteria* were represented by *Pseudomonas* spp., selected strains also included one strain of an unusual lignocelluloytic-related genus, *Enterobacter* sp. AR451.

The isolate identified as *Achromobacter* sp. AR 476-2 showed poor growth in mVL70 medium and subtle halos of hydrolysis in CMC-Congo Red agar. However, when grown in DTSB–CMC during 96 h, it was able to reach CMCase titles of 0.23 ± 0.06 IU ml^{-1} (Table 1).

Finally, representatives of the phylum *Bacteroidetes* were isolated from samples of industrial liquor. The isolates AR11, AR13, and AR126 displayed low endoglucanase activity in the conditions here assayed (Table 1), yet higher xylanase activity reaching up to 1.2 IU ml^{-1} was observed (data not shown).

Discussion

This study focuses on the isolation and selection of bacteria with potential for modification and decomposition of lignocellulosic biomass. The main industrial activity in the Northwest of Argentina is sugarcane-based, where bioethanol is produced from molasses. However, the use of lignocellulosic agricultural residues, as well as other non-food plant biomass, is conceived to contribute to a bio-based economy. Herein, a collection of cellulose-degrading bacteria was preliminarily characterized from samples collected locally, in order to assess and select microorganisms with potential application in the biorefinery context of the local industry.

An enrichment-isolation procedure was applied, which allowed the isolation of 233 bacterial strains using aerobic conditions. Most of the isolates were obtained from cultures incubated at 30 °C, even though cultivation assays at 45 °C were carried out. However, only a few colonies developed at 45 °C (data not shown). Also, a few yeast colonies and only one filamentous fungus were recovered under the cultivation conditions here employed; consequently, bacteria were almost exclusively retrieved.

A large fraction of the isolates (>40%) was obtained from enrichments using a modified VL70 medium

(Fig. 1A). According to this, the VL medium has proven to be useful for the isolation of bacteria from various sources, despite the fact that was primarily formulated for soil bacteria [13]. Employing classic microbiological criteria, a subset of bacterial isolates was selected according to their cellulolytic potential, such as production of prominent halo of cellulose in petri dish and/or evidence of filter paper degradation (Table 1). These isolates were taxonomically related to four major phyla: *Firmicutes* (62.2%), *Proteobacteria* (24.4%), *Actinobacteria* (6.7%), and *Bacteroidetes* (6.7%) (Fig. 3). It is important to bear in mind that the taxonomic distribution observed in this work is not necessarily associated with the bacterial diversity in the samples analyzed. The culture-based approach used here led us to obtain strains restricted to the readily cultivable ones, and so key components of the samples and enrichment cultures might have been missed in our work. Other culture-dependent studies utilizing similar enrichment procedures reported the dominance of *Firmicutes* and a comparable taxonomic distribution for soil samples [36], guts of xylophagous insects [10, 30], and compost [37]. Among the most frequent genera obtained, *Bacillus* spp. includes aerobic or facultative anaerobic, rod-shaped bacteria with a wide diversity with respect to carbon source utilization and growth conditions. The prevalence of this genus may be a sign of their numerical abundance; nevertheless, bias due to the procedures and culture media used must be considered. Also belonging to *Firmicutes*, members of *Paenibacillaceae* family have been often described as strong cellulolytic and hemicellulolytic bacteria, reports which are in agreement to our observations [22–26]. However, to our knowledge there are no previous reports of a zymographic profile revealed with CMC as substrate for *Cohnella* species.

The isolates here obtained that were grouped into *Staphylococcus* genus represented the 4.4% of the selected strains (Fig. 3). Cellulase activity is not a property commonly found among staphylococci; however, cellulolytic strains related to this genus have been described from gut of xylophagous termites [10].

The isolates belonging to *Actinobacteria* phylum obtained here exhibited scarce enzymatic activity on CMC; but they produced filter paper degradation (Table 1) and xylanases. Several actinobacteria involved in cellulose and xylan degradation have been described from a variety of environmental samples such as compost, soil, and from the guts of xylophagous insects [5, 27]. It was also suggested that the participation in cellulose digestion in the termite gut by *Microbacterium* spp. and other actinobacetria [22, 28].

Regarding *Proteobacteria*, several species of *Pseudomonas* spp. isolated from insect guts have been extensively described due to its potential for industrial use in the degradation of lignocellulosic biomass [10, 29, 30]. The selected isolates belonging to this genus exhibited promising cellulolytic and hemicellulolytic activities as well as evidence of mild FP degradation as is shown in Table 1. *Enterobacter* sp. AR451, isolated from *Coleoptera* larvae (Table 1), showed significant FP degradation in spite of the fact that cellulase activity has occasionally been reported for a few members of the *Enterobacteriaceae* family [10]. Moreover, members of *Enterobacter* spp. have not been considered relevant regarding degradation of lignocelluloses until recent culture-dependent reports, which described strains of this genus with high lignocellulolytic potential [11, 31].

It is noticeable that the literature relating to cellulases and hemicellulases with *Achromobacter* species describe their presence as members of microbial consortia. A consortium including *A. xylosoxidans* and *Fusarium sporotrichioides* that produced CMCase and xylanase titles of $0.24\,\mathrm{IU\,ml^{-1}}$ and $3.75\,\mathrm{IU\,ml^{-1}}$, respectively, was described by Yang and co-workers [32]. Nevertheless, the authors conclude that their results do not contain sufficient evidence to state the role of *A. xylosoxidans* in the assayed system. Similar observations were reported by Ghio et al. [33] who proposed that *Achromobacter* spp. lack the key enzymes for the degradation of lignocellulosic substrates. Thus, the isolation of pure strains as we performed, would contribute to describe if *Achromobacter* species are able to produce cellulolytic or xylanolytic enzymes, or if species of this genus utilize the sugars released by the degradation at the expense of other partners in complex cultures.

Finally, few reports have been found regarding cellulase production on genera such as *Chryseobacterium* spp. and *Sphyngobacterium* spp. However, it is not surprising that members of this phylum are capable of biodegradation of organic compounds since can be found ubiquitously in the environment [29, 34, 35]. Hence, the strains isolated have an interesting potential for hydrolytic activities toward celluloses and hemicelluloses.

Most of the isolates displayed poor CMCase activity and a few of them showed evidence of mild FP degradation in the general screening conditions used here (Table 1). Concerning this, it is important to draw attention to the fact that all isolates may vary in growth capacity, which would make difficult the comparison and quantification of cellulolytic activities in a fair manner. In this work, all isolates were grown in nutritive medium supplemented with CMC in order to maximize biomass for detection of enzymatic activity. Still,

cellulases and hemicellulases production may be dependent upon culture conditions.

Major bottlenecks in screening projects like this are: (i) the need of the optimization of the media culture composition and the growth conditions for each isolate in order to a better evaluation of the enzymatic activity, and (ii) the fact that the methods to select enzymes that catalyze the desired reaction are frequently underdeveloped. As was also mentioned before [27], bacterial strains taxonomically related to the ones evaluated here are able to produce enzymes differently: some may secrete enzymes to the culture broth, while others may harbor enzymes on the cell surface or internally, and still some cellulases may end up in solution from cell lysis. Yet, despite those limitations and the bias associated with cultivation, pure strains can provide insights of the physiological behavior which molecular-based analysis alone cannot. As a result, promising strains selected here will be subjected to further improvement of growth and enzyme production, as well as molecular studies to gain insights on the microbial mechanisms to biomass deconstruction.

Acknowledgments

This research was supported by Argentinean grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT). A.P.M. thanks CONICET for doctoral fellowship. The paper does not contain human studies or experiments using animals.

Conflicts of interest

All authors declare no financial or commercial conflicts of interest.

References

[1] Lambertz, C., Garvey, M., Klinger, J., Heesel, D., et al., 2014. Challenges and advances in the heterologous expression of cellulolytic enzymes: a review. Biotechnol. Biofuels, **7**, 135.

[2] Wilson, D.B., 2008. Three microbial strategies for plant cell wall degradation. Ann. NY Acad. Sci., **1125**, 289–297.

[3] Bornscheuer, U., Buchholz, K., Seibel, J., 2014. Enzymatic degradation of (ligno) cellulose. Angew. Chem. Int. Ed. Engl., **53**, 10876–10893.

[4] DeBoy, R.T., Mongodin, E.F., Fouts, D.E., Tailford, L.E., et al., 2008. Insights into plant cell wall degradation from the genome sequence of the soil bacterium *Cellvibrio japonicus*. J. Bacteriol., **190**, 5455–5463.

[5] Okeke, B.C., Lu, J., 2011. Characterization of a defined cellulolytic and xylanolytic bacterial consortium for bioprocessing of cellulose and hemicelluloses. Appl. Biochem. Biotechnol., **163**, 869–881.

[6] Wongwilaiwalin, S., Laothanachareon, T., Mhuantong, W., et al., 2013. Comparative metagenomic analysis of microcosm structures and lignocellulolytic enzyme systems of symbiotic biomass-degrading consortia. Appl. Microbiol. Biotechnol., **97**, 8941–8954.

[7] Gronenberg, L.S., Marcheschi, R.J., Liao, J.C., 2013. Next generation biofuel engineering in prokaryotes. Curr. Opin. Chem. Biol., **17**, 462–471.

[8] Wang, M., Li, Z., Fang, X., Wang, L., Qu, Y., 2012. Cellulolytic enzyme production and enzymatic hydrolysis for second-generation bioethanol production. Adv. Biochem. Engin. Biotechnol., **128**, 1–24.

[9] Do, T.H., Nguyen, T.T., Nguyen, T.N., Le, Q.G., et al., 2014. Mining biomass-degrading genes through Illumina-based de novo sequencing and metagenomic analysis of free-living bacteria in the gut of the lower termite Coptotermes gestroi harvested in Vietnam. J. Biosci. Bioeng., **118**, 665–671.

[10] Pourramezan, Z., Ghezelbash, G.R., Romani, B., Ziaei, S., et al., 2012. Screening and identification of newly isolated cellulose_degrading bacteria from the gut of xylophagous termite *Microcerotermes diversus* (Silvestri). Mikrobiologiia, **81**, 736–742.

[11] DeAngelis, K.M., Sharma, D., Varney, R., BlakeSimmons, B., et al., 2013. Evidence supporting dissimilatory and assimilatory lignin degradation in *Enterobacter lignolyticus* SCF1. Front. Microbiol., **4**, 1–14. DOI: 10.3389/fmicb.2013.00280

[12] Gardner, J.G., Crouch, L., Labourel, A., Forsberg, Z., et al., 2014. Systems biology defines the biological significance of redox-active proteins during cellulose degradation in an aerobic bacterium. Mol Microbiol., **94**, 1121–1133.

[13] Schoenborn L., Yates, P.S., Grinton, B.E., Hugenholtz, P, et al., 2004. Liquid serial dilution is Inferior to solid media for isolation of cultures representative of the phylum-level diversity of soil bacteria. Appl. Environ. Microbiol., **70**, 4363–4366.

[14] Gupta, P., Samant, K., Sahu, A., 2012. Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. Int. J. Microbiol., Article ID 578925, 1–5. DOI: 10.1155/2012/578925

[15] Miller, G.L., 1959. Use of dinitrosaIicyIic acid reagent for determination of reducing sugar. Anal. Chem., **31**, 426–428.

[16] Bailey, M.J., 1995. Extraction of DNA from the phyllosphere. In: Nucleic Acids in the Environment: Methods and Applications (Trevors, J.T. and van Elsas, J.D., eds.), Springer-Verlag, Berlin, 89–109.

[17] Martínez, M.A., Delgado, O.D., Breccia, J.D., Baigorí, M.D., et al., 2002. Revision of the taxonomic position of the xylanolytic *Bacillus* sp. MIR32 reidentified as *Bacillus halodurans* and plasmid-mediated transformation of *B. halodurans*. Extremophiles, **6**, 391–395.

[18] Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., et al., 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int. J. Syst. Evol. Microbiol., **62**, 716–721.

[19] Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M., et al., 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verruco microbia*. Appl. Environ. Microbiol., **68**, 2391–2396.

[20] Koroiva, R., Souza, C.W., Toyama, D., Henrique-Silva, F., et al., 2013. Lignocellulolytic enzymes and bacteria associated with the digestive tracts of *Stenochironomus* (Diptera: Chironomidae) larvae. Genet. Mol. Res., **12**, 3421–3434.

[21] Romano, N., Gioffré, A., Sede, S.M., Campos, E., et al., 2013. Characterization of cellulolytic activities of environmental bacterial consortia from an Argentinian native forest. Curr. Microbiol., **67**, 138–147.

[22] Wenzel, M., Schönig, I., Berchtold, M., Kämpfer, P., et al., 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. J. Appl. Microbiol., **92**, 32–40.

[23] Waeonukul, R., Kyu, K.L., Sakka, K., Ratanakhanokchai, K., 2009. Isolation and characterization of a multienzyme complex (cellulosome) of the *Paenibacillus curdlanolyticus* B-6 grown on Avicel under aerobic conditions. J. Biosci. Bioeng., **107**, 610–614.

[24] St John, F.J., Preston, J.F., Pozharski, E., 2012. Novel structural features of xylanase A1 from *Paenibacillus* sp. JDR-2. J. Struct. Biol., **180**, 303–311.

[25] Hameed, A., Hung, M.H., Lin, S.Y., Hsu, Y.H., et al., 2013. *Cohnella formosensis* sp. nov., a xylanolytic bacterium isolated from the rhizosphere of Medicago sativa L. Int. J. Syst. Evol. Microbiol., **63**, 2806–2812.

[26] Fukuda, M., Watanabe, S., Yoshida, S., Itoh, H., et al., 2010. Cell surface xylanases of the glycoside hydrolase family 10 are essential for xylan utilization by *Paenibacillus* sp. W-61 as generators of xylo-oligosaccharide inducers for the xylanase genes. J. Bacteriol., **192**, 2210–2219.

[27] Maki, M.L., Broere, M., Leung, K.T., Qin, W., 2011. Characterization of some efficient cellulase producing bacteria isolated from paper mill sludges and organic fertilizers. Int. J. Biochem. Mol. Biol., **2**, 146–154.

[28] Berlemont, R., Martiny, A.C., 2013. Phylogenetic distribution of potential cellulases in bacteria. Appl. Environ. Microbiol., **79**, 1545–1554.

[29] Maki, M.L., Idrees, A., Leung, K.T., Qin, W., 2012. Newly isolated and characterized bacteria with great application potential for decomposition of lignocellulosic biomass. J. Mol. Microbiol. Biotechnol., **22**, 156–166.

[30] Tarayre C., Brognaux, A., Bauwens, J., Brasseur, C., et al., 2013. Isolation of amylolytic, xylanolytic, and cellulolytic microorganisms extracted from the gut of the termite *Reticulitermes santonensis* by means of a micro-aerobic atmosphere. World J. Microbiol. Biotechnol., **30**, 1655–1660.

[31] Jiménez, D.J., Korenblum, E., van Elsas, J.D., 2014. Novel multispecies microbial consortia involved in lignocellulose and 5-hydroxymethylfurfural bioconversion. Appl. Microbiol. Biotechnol., **98**, 2789–2803.

[32] Yang, H., Wu, H., Wang, X., Cui, Z., et al., 2011. Selection and characteristics of a switchgrass-colonizing microbial community to produce extracellular cellulases and xylanases. Bioresour. Technol., **102**, 3546–3550.

[33] Ghio, S., Lorenzo, G.S., Lia, V., Talia, P., et al., 2012. Isolation of *Paenibacillus* sp. and *Variovorax* sp. strains from decaying woods and characterization of their potential for cellulose deconstruction. Int. J. Biochem. Mol. Biol., **3**, 352–364.

[34] Cho, M.J., Kim, Y.H., Shin, K., Kim, Y.K., et al., 2010. Symbiotic adaptation of bacteria in the gut of *Reticulitermes speratus*: low endo-beta-1,4-glucanase activity. Biochem. Biophys. Res. Commun., **395**, 432–435.

[35] Kabisch, A., Otto, A., König, S., Becher, D., et al., 2014. Functional characterization of polysaccharide utilization loci in the marine *Bacteroidetes* '*Gramella forsetii*' KT0803. ISME J., **8**, 1492–1502.

[36] Rastogi G., Muppidi, G.L., Gurram, R.N., Adhikari, A., et al., 2009. Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. J. Ind. Microbiol. Biotechnol., **36**, 585–598.

[37] Wang, W., Yan, L., Cui, Z., Gao, Y., et al., 2011. Characterization of a microbial consortium capable of degrading lignocellulose. Bioresour. Technol., **102**, 9321–9324.

