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Toward Plant Cell Wall Degradation Under Thermophilic Condition: A Unique Microbial Community Developed Originally from Swine Waste

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Abstract A unique thermophilic microbial community developed initially from swine waste was investigated in this study. Cellulase activities were observed when this community was inoculated to media containing either cellulose or carboxymethylcellulose at 57 °C. Through constructing a clone library for the 16S ribosomal DNA, it was revealed that this community was mainly composed of three genera: *Thermobacillus*, *Brevibacillus*, and *Anoxybacillus*. New findings regarding the thermo- and pH stability of crude cellulases secreted by *Brevibacillus* sp. JXL were presented. Recent study on the growth characteristics of *Anoxybacillus* sp. 527 was discussed.

Keywords Cellulose · Thermophilic · Cellulase · Microbial community · *Brevibacillus* sp. JXL · *Anoxybacillus* sp. 527

Introduction

Lignocellulose is the most abundant and renewable natural resource and substrate available for conversion to fuels [1]. Based on the most recent report [2], in the near term, more than 1.3 billion tons of biomass could be produced annually in the USA on a sustainable basis, mostly from agricultural and forestry sources. However, due to the recalcitrance of natural lignocellulosic feedstocks, pretreated biomass must be hydrolyzed by enzymes to produce sugars [1, 3, 4]. During recent decades, even though huge efforts have been devoted to improving the activities of the cellulases and reducing the cost for enzyme production, cellulases are still expensive enzymes. It has been reported by Novozyme Biotech and Genencor International that the cellulase cost for producing 1 gal ethanol from cellulose was reduced from $4.5 to approximately 20 cents. But this claim has not been widely accepted since the cellulase mixture was tested only for the specifically pretreated cellulosic substrate and cannot be simply applied to other biomass [5].

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Currently, most pretreatment techniques require high temperatures (more than 100 °C) to disrupt rigid plant cell wall structures. Despite the fact that cellulases are commercially available, most fungal cellulases have optimal activities at 50 °C. Therefore, pretreated biomass must be cooled down first to meet this temperature requirement. This cooling step adds cost and lengthens the treatment time. Therefore, thermostable cellulases produced by thermophilic microbes are eagerly sought due to their advantages: (1) these cellulases can retain their activities at higher temperatures during prolonged reactions [6], (2) cooling problems for pretreated biomass can be simplified [1], and (3) potential contamination can be easily prevented [1, 7] when thermophiles are used.

As described by Blackburn [8], a pilot plant of 3.8 m^3 was built several years ago to treat swine waste from a swine-finishing building at Southern Illinois University Carbondale using aerobic and thermophilic (57 °C) degradation process. Lyophilized sample from this reactor served as the source for thermophilic microorganisms. From this source sample, we have already isolated and identified two cellulolytic bacteria, *Brevibacillus* sp. JXL and *Anoxybacillus* sp. 527 [9, 10]. Both of them are aerobic and thermophilic and can utilize a variety of carbohydrates. In this study, we present the cellulose-degrading potential and the composition of the microbial community existing in the source sample. Additionally, we report more recent data on the thermo- and pH stability for strain JXL and growth characteristics regarding strain 527.

Materials and Methods

Chemicals

SigmaCell cellulose (50 μm), glucose, xylose, xylan, and carboxymethylcellulose (CMC) were bought from Sigma Aldrich (St. Louis, MO, USA). Yeast extract, tryptone, agarose, and peptone were purchased from Difco laboratories (Sparks, MD, USA). All other chemicals used in this study were of the highest grade possible from Fisher Scientific (Pittsburgh, PA, USA), if not noted specifically.

Capability for Cellulose Degradation

To evaluate the cellulose-degrading potential of this bacterial source sample, we took 0.5 g and added it to 50 ml 0.85% NaCl. After the mixture was shaken on a rotary shaker for 1 h at 57 °C, 10 ml sample was supplemented to 90 ml of two different media. One medium referred to as cellulolytic contained (per liter): $NaHCO_3$ (2.06 g), NH_4Cl (0.68 g), K_2HPO4 (0.296 g), KH_2PO4 (0.18 g), $(NH_4)_2SO_4$ (0.15 g), $MgSO_4.7H_2O$ (0.12 g), $CaCl_2.2H_2O$ (61.0 mg), $FeSO_4.7H_2O$ (21.0 mg), NaCl (10.0 mg), $MnSO_4.H_2O$ (5.0 mg), $CoCl_2.H_2O$ (1.0 mg), $ZnSO_4.7H_2O$ (1.0 mg), $CuSO_4.5H_2O$ (0.1 mg), H_3BO_3 (0.1 mg), $KAl(SO_4)_2.12H_2O$ (0.1 mg), $Na_2MoO_4.2H_2O$ (0.1 mg), L-cysteine–$HCl.H_2O$ (0.25 g), and Wolfe's vitamin solution (American Type Culture Collection, 10 ml) [11]. Another one was called basal medium, which was composed of (per liter): 0.004 g $FeCl_3$, 1 g $(NH_4)_2SO_4$, 0.6 g NaCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KH_2PO_4, and 0.002 g $CaCl_2.2H_2O$. To the cellulolytic or basal medium, SigmaCell cellulose or CMC was added to make the final concentration as 1%, respectively. For each substrate, three replicates were set up in 250-ml Erlenmeyer flasks and incubated at 57 °C on a rotary shaker set at 170 rpm. At different time points, 1 ml sample was taken out and centrifuged at 18,000×*g* for 10 min. The supernatant which was also mentioned as crude cellulase solution was utilized for cellulase activity assay. This assay was based on

Ghose's procedure to measure reducing sugars released from filter paper degradation using dinitrosalicylic acid reagent adopting glucose as a standard [12]. One unit of filter paper activity (FPA) was defined as 1 μmol glucose equivalents formed per milliliter per minute. For this experiment, the activity tests were conducted using citrate buffer (50 mM, pH 4.8) at 50 °C.

Construction and Sequencing of 16S rDNA Gene Clone Libraries

In order to decipher the microbial community structure of the bacterial source sample, 0.1 g subsample was weighed for microbial DNA extraction using a Powersoil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA). The 16S ribosomal DNA (rDNA) in the obtained genomic DNA was amplified by polymerase chain reaction (PCR) following a program reported by Liang [9]. Briefly, PCR was conducted in a 50-μl reaction mixture containing (final concentration): 1 μl of extracted DNA, 1× PCR buffer (Promega, Madison, WI, USA), 2.5 mM MgSO4, 0.2 mM dNTPs mixture, 2.5 U Taq DNA polymerase (Promega), and 10 μM of each of the forward and reverse primers. PCR amplification was carried out according to the following protocol: initial denaturation for 5 min at 95 °C followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 1 min. A final elongation step at 72 °C for 30 min was included. PCR products were analyzed by gel electrophoresis. The 16S rDNA band on the agarose gel was cut and purified by a Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA). The purified DNA was used for constructing a clone library employing a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommended procedures. Plasmids carrying the correct inserts were extracted from positive transformants by a Qiagen plasmid extraction kit and sent for sequencing by Agencourt Biosciences (Beverly, MA, USA). Twelve clones were sequenced with excellent readings.

Nucleotide Sequence

The partial 16S rDNA sequences of the 12 clones have been submitted to GenBank. The accession numbers are from GQ471925 to GQ471936.

Isolation and Identification of Cellulose Degraders

After the bacterial source sample grew in cellulolytic medium containing cellulose for 2 days as described above, 100 μl of the culture was spread onto cellulose plate made from the cellulolytic medium with the presence of agar (15 g l^{-1}). After the plates were incubated at 57 °C for 1 day, colonies started to appear. To choose the colonies with the highest cellulose hydrolyzing potential, Congo red (0.1%) was used to flood the plate for 15 min. Plate was then destained by NaCl (1 M) for clear zone visualization. Two colonies were selected for further purification by transferring to cellulose plate five times. Purified colonies were identified as *Brevibacillus* sp. JXL [9] and *Anoxybacillus* sp. 527 [10] based on 16S rDNA sequences.

Thermostability and pH Stability for Strain JXL

Strain JXL has been demonstrated to have optimal enzymatic activities at 50 °C and pH 7.0 when cells are grown on cellobiose [9]. To test whether the crude cellulases have different optimal temperature when grown on a different substrate, we grew the cells using glucose (1%) as a carbon source. The FPA assay was conducted at pH 7.0 with temperatures ranging from 50 to 100 °C.

To determine how stable the cellulase activities are, we incubated the enzyme solution in citrate buffer (pH 7.0) at different temperatures spanning from 50 to 100 °C for 3 h. At different times, 1 ml of the enzyme solution was taken and tested for FPA. We also examined the residual activity of the cellulases after allowing the enzymes to be incubated at room temperature in different buffers with different pHs for 24 h. The pH ranged from 5 to 10. Between pH 5 and 7, phosphate buffer was used. Between pH 8 and 10, citrate buffer was employed.

Growth of Strain 527 with Different Substrates

To assess the growth of strain 527 with different substrates, cells were initially grown in Luria–Bertani broth (LB) medium containing (per liter): 10 g yeast extract, 5 g tryptone, and 10 g NaCl. Following 8 h growth in this medium, 10 ml of the culture was inoculated into 90 ml of medium composed of (per liter): 1.0 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.5 g L-asparagine, 0.5 g KCl, 0.5 g yeast extract, 0.2 g $MgSO_4$, and 0.1 g $CaCl_2$ [11]. Several carbohydrates including glucose, xylose, xylan, CMC, and cellulose were examined. To prevent sugar carmelization, stock solution (10%) for each substrate was separately autoclaved from the medium. The final concentration for each substrate was 1%.

Results and Discussions

The microbial community in the bacterial source sample demonstrated cellulose hydrolyzing capability as shown by Fig. 1. During the first 12 days, the crude cellulases from cultures with cellulose or CMC had similar activities. But after that time, enzymes from CMC-grown culture had much higher activities. As a matter of fact, the cellulase activities in these two cultures, 0.02 U ml^{-1} for cellulose and 0.04 U ml^{-1} for CMC, were similar to what have been reported for strain JXL and 527 [9, 10]. However, these two values were obtained under a condition which may not be optimal. Based on our experience working with strain JXL and 527, enzymatic activities can be enhanced dramatically once the optimal temperature, pH, and buffer conditions were provided.

Corresponding to the increased enzyme activities, the released reducing sugars in the CMC-grown culture also increased with time, which served as a direct evidence for CMC degradation. In particular, since no other carbon sources or any other nutrients, like yeast extract or vitamins, were supplied to the basal medium, the microbial community must be able to utilize CMC as sole carbon and energy sources.

Inspired by the great potential of this source sample, we were curious about what other bacteria may be present in the community besides the two that were already isolated. Hence, we went ahead and constructed a 16s rDNA clone library for the extracted DNA. Twelve clones were sent for sequencing. The phylogenetic analysis was accomplished by comparing the sequences obtained with entries in GenBank database using basic local alignment search tool. As shown in Fig. 2, ten clones were closely related to *Thermobacillus composti* strain KWC4 and *Thermobacillus xylanilyticus*. Strain KWC4 is a Gram-negative, rod-shaped, spore-forming, and moderately thermophilic bacterium isolated from a composting reactor and grows optimally at 50 °C [13]. *T. xylanilyticus* is an aerobic, thermophilic, and xylanolytic spore-forming bacterium and was isolated from farm soil situated underneath a manure heap in Northern France [14]. It grows optimally at 63 °C. In addition to glucose, this isolate utilizes xylose, arabinose, mannose, cellobiose, galactose, maltose, sucrose, xylan, and starch. As revealed by 16S rDNA sequences, strain

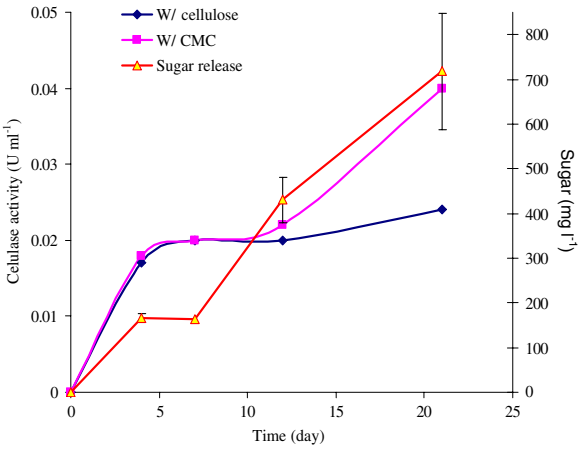

Fig. 1 Cellulase activities measured as filter paper unit (units per milliliter) for crude enzyme solutions from cultures with either cellulose (*diamonds*) or carboxymethylcellulose (CMC; *squares*). Reducing sugars released from CMC hydrolysis is presented as milligram per liter (*triangles*). Assays were conducted at 50 °C in citrate buffer (pH 4.8). The bacterial source sample served as the inoculum. *Error bars* denoted standard deviations from means of three replicates

KWC4 is most closely related to *T. xylanilyticus* with 95.7% similarity. Based on the classification provided by ribosomal database project, these ten clones are 100% *Thermobacillus*. The other two clones were mostly related to strain JXL and 527, respectively. Thus, the source sample contains a group of *Bacillus* species that are thermophilic and able to use a broad range of carbohydrates.

The simplicity of the community structure is mainly due to the process of developing this source sample. As described in details by Blackburn [8], the source sample was initially attained from swine-waste treatment process. Then, the sample was used consecutively to ferment corn fiber in distillers' dried grain (DDG). DDG typically consists of arabinoxylan, cellulose, and adherent starch [6]. Therefore, a unique cluster of *Bacillus* genus able to thrive at 57 °C and grow on these carbohydrates remained in this source sample.

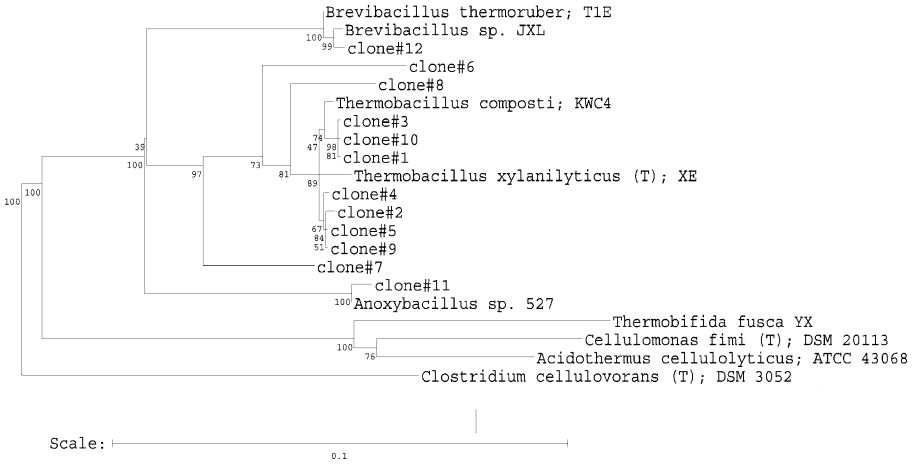

Fig. 2 Phylogenetic tree based on 16S rDNA sequences of the 12 clones and the two isolates, *Brevibacillus* sp. JXL and *Anoxybacillus* sp. 527, their closest relatives, and a selected group of cellulose-degrading microbes (sequences from the Ribosomal Database Project release 10). The *tree* was constructed using Weighbor tree method (a distance corrected modification of Jukes Cantor). *Numbers at the nodes* indicate bootstrap values based on 100 replicates, while the *scale* indicates number of changes per nucleotide position

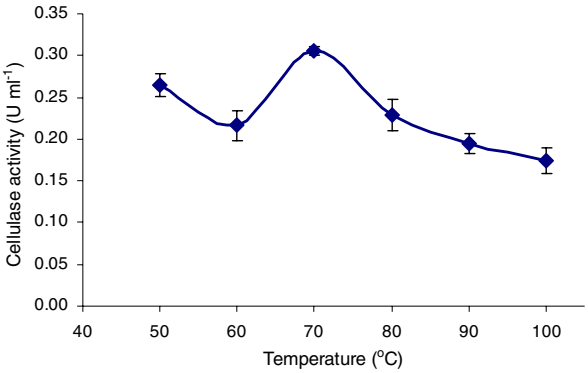

Fig. 3 Effect of temperature on crude cellulase activities for enzymes secreted by *Brevibacillus* sp. JXL grown on glucose. Cellulase activity (units per milliliter) assay was conducted at pH 7.0 with temperatures ranging from 50 to 100 °C. *Error bars* represented standard deviations from means of three replicates

As reported by Liang [9], strain JXL is aerobic and thermophilic. It can grow on crystalline cellulose, xylan, glucose, cellobiose, and xylose at 57 °C with a medium pH 7.0. It produces extracellular cellulases and has protuberant cellulosome-resembling structures at cell surface. The crude cellulases from cells grown on cellobiose possess optimal activities at 50 °C and pH range of 7 to 9. The crude cellulases from cells grown on glucose indicated a relatively different temperature preference as shown in Fig. 3. For these enzymes, activities at 70 °C were higher than those at 50 °C. The possible explanation for this difference could be that different sets of enzymes were induced by different substrates. Similar phenomenon was described for *Thermobifida fusca* as different cellulase profiles were observed with different carbohydrates [6]. A common characteristic between enzymes induced by cellobiose and glucose is that cellulases retained their activities even at 90 and 100 °C for 1 h.

The thermostability test conducted for 3 h confirmed this observation. As presented in Fig. 4, when the crude enzymes were kept between 50 and 80 °C, more than 60% of activities were retained after 3 h incubation. At 90 and 100 °C, enzymes lost their activities

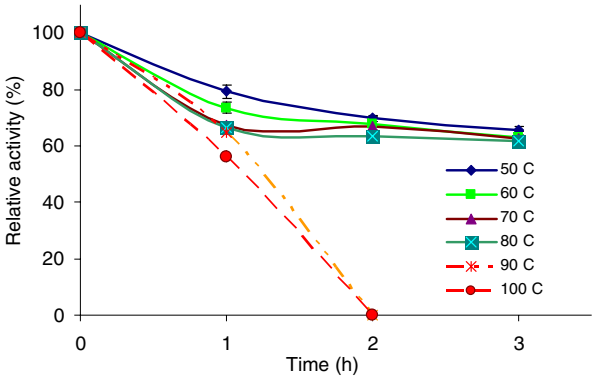

Fig. 4 Effect of incubation of crude cellulases at different temperatures on residual activities. Enzyme solution was taken from *Brevibacillus* sp. JXL culture grown with cellulose and glucose. Relative activities were expressed as the percentage of remaining activities at different time points to the original value. *Error bars* represented standard deviations from means of three replicates

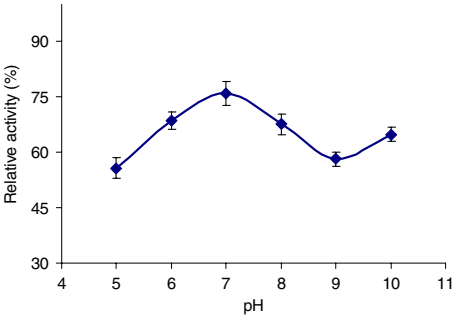

Fig. 5 Effect of incubation of crude cellulases at different pH on residual activities. Enzyme solution was taken from *Brevibacillus* sp. JXL culture grown with cellulose and glucose. Relative activities were expressed as the percentage of remaining activities at different time points to the original value. *Error bars* represented standard deviations from means of three replicates

after 2 h. In contrast, cellulases secreted by *Clostridium thermocellum* have no activities at 80 °C [15]. The best commercial cellulase enzymes from Genencor, ACCELLERASE® 1500, have optimal activities between 50 and 65 °C, and their activities can be easily inactivated at 70 °C (www.genencor.com). In terms of pH stability, the cellulases retained 75% of their activities after sitting at room temperature in pH 7 buffer overnight. Under the same conditions, approximately 50% of activities were lost when pH 5 and 9 buffers were used (Fig. 5). The excellent stability with regard to temperature and pH demonstrated the potential use of these enzymes in industrial scale applications related to plant material saccharification.

The genus of *Anoxybacillus* was first introduced by Pikuta [16]. As its name applied, it has been considered as small rod bacillus with no need of oxygen for living or obligate anaerobes. In 2003, the description of this genus was corrected to be aerotolerant anaerobes or facultative anaerobes [17]. Besides the ten species listed by Gul-Guven [18], another new species as *Anoxybacillus bogrovensis* was reported very recently [19]. Among the 11 species, it has been proven that five of them, such as *Anoxybacillus kamchatkensis* DSM 14988 [20], *Anoxybacillus flavithermus* DSM 2641 [16], *Anoxybacillus contaminans* DSM 15866 [21], *Anoxybacillus amylolyticus* DSM 15939 [22], and *Anoxybacillus rupiensis* DSM 17127 [23], are aerobes. In terms of strain 527, we have observed that it grew rapidly under aerobic conditions. As shown in Fig. 6, the cells started logarithmic phase after 5 h in LB medium. In contrast, when oxygen was limited, much slower growth was resulted.

Regarding substrate, especially carbohydrate utilization, large variance exists for the 11 species of *Anoxybacillus*. Glucose cannot be utilized by three species, including *A. kamchatkensis* strain KG8 [18], *A. flavithermus* [16], and *A. amylolyticus* [22]. In the case

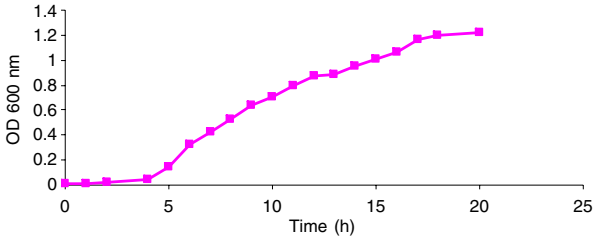

Fig. 6 A typical growth curve of *Anoxybacillus* sp. 527 in LB medium. We have observed this curve repeatedly

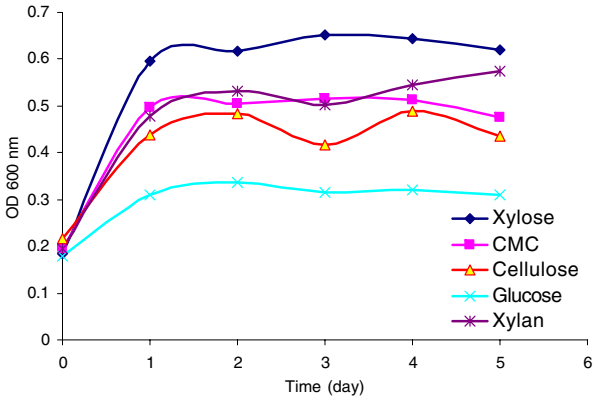

Fig. 7 Representative growth curves of *Anoxybacillus* sp. 527 on different carbohydrates from our multiple experiments

of xylose, these three species plus *A. kamchatkensis* [20], *A. bogrovensis* [19], and *Anoxybacillus kestanbolensis* [24] cannot grow on it. Our test of the growth of strain 527 on different carbohydrates indicated that it could utilize xylose, CMC, xylan, cellulose, and glucose besides cellobiose, lactose, and sucrose which are already reported [10]. But the growth rate on glucose was the lowest (Fig. 7).

Table 1 Comparison of the growth and substrate utilization characteristics of *Anoxybacillus* sp. 527 with related species.

Characteristic	1	2	3	4
Optimal temperature for growth(°C)	57	55	55	61
Optimal pH for growth	7	7.0	6.0–6.5	5.6
Oxygen	Strictly aerobe	Strictly aerobe	Strictly aerobe	Facultative anaerobic
Substrate used				
Cellobiose	Y	NA	NA	NA
Cellulose	Y	NA	NA	NA
CMC	Y	NA	NA	NA
Fructose	NA	NA	NA	NA
Galactose	NA	N	Y	Y
Glucose	Y	Y	Y	Y
Lactose	Y	N	NA	N
Maltose	NA	NA	N	N
Mannose	NA	NA	NA	NA
Starch	NA	Y	NA	Y
Sucrose	Y	N	Y	Y
Trehalose	NA	NA	Y	Y
Xylan	Y	NA	NA	N
Xylose	Y	Y	Y	N

Data from several publications including [18, 22, 23, 25] were combined and presented here

1 Anoxybacillus sp. 527, *2 Anoxybacillus rupiensis* R270^T DSM 17127^T, *3 Geobacillus tepidamans* GS5-97^T DSM 16325^T, *4 Anoxybacillus amylolyticus* MR3CT DSM 15939^T, *Y* positive, *N* negative, *NA* not available

Reviewing of the 16S rDNA sequences of the strain 527 (FJ744749) revealed that it is: (1) 99% identical to a newly proposed species/strain: *Anoxybacillus beppuensis* strain TSSC-1 (EU710556.1) which has no detailed information available; (2) 99% identical to *A. rupiensis* (AJ879076.1) which is a thermophilic, strictly aerobic, Gram-positive, and spore-forming hemo-organotrophic bacterium isolated from hot springs in the region of Rupi basin, Bulgaria as producers of amylolytic enzymes [23]; (3) 97% identical to a *Geobacillus tepidamans* sp. nov., GS5-97T (AY563003.1) isolated from a beet sugar factory in Leopoldsdorf, Lower Austria. This strain is also moderately thermophilic, strictly aerobic, Gram-positive, motile, and spore-forming [25]; and (4) 97% identical to *A. amylolyticus* (AJ618979.1) isolated from geothermal soil at Antarctica and is a thermophilic, Gram-positive, and facultative anaerobe [22]. Therefore, strain 527 is highly related to a group of thermophilic *Bacillus* distributed around the world.

Strain 527 was compared with the aforementioned related strains in terms of growth conditions and substrate utilization. As shown in Table 1, strain 527 is the only one that can hydrolyze large recalcitrant polymers, such as crystalline cellulose, xylan, and CMC. Based on the features of the other three strains, strain 527 is possibly able to degrade starch as well. Verification of this hypothesis is ongoing in our lab.

Overall, the bacterial source sample obtained through swine-waste treatment and enriched by aerobic and thermophilic DDG degradation process is composed of a unique microbial community possessing great potential toward plant cell wall degradation.

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