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ORIGINAL PAPER

Study of cellulases from a newly isolated thermophilic and cellulolytic *Brevibacillus* sp. strain JXL

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Abstract A potentially novel aerobic, thermophilic, and cellulolytic bacterium designated as *Brevibacillus* sp. strain JXL was isolated from swine waste. Strain JXL can utilize a broad range of carbohydrates including: cellulose, carboxymethylcellulose (CMC), xylan, cellobiose, glucose, and xylose. In two different media supplemented with crystalline cellulose and CMC at 57°C under aeration, strain JXL produced a basal level of cellulases as FPU of 0.02 IU/ml in the crude culture supernatant. When glucose or cellobiose was used besides cellulose, cellulase activities were enhanced ten times during the first 24 h, but with no significant difference between these two simple sugars. After that time, however, culture with glucose demonstrated higher cellulase activities compared with that from cellobiose. Similar trend and effect on cellulase activities were also obtained when glucose or cellobiose served as a single substrate. The optimal doses of cellobiose and glucose for cellulase induction were 0.5 and 1%. These inducing effects were further confirmed by scanning electron microscopy (SEM) images, which indicated the presence of extracellular protuberant structures. These cellulosome-resembling structures were most abundant in culture with glucose, followed by cellobiose and without sugar addition. With respect to cellulase activity assay, crude cellulases had an optimal temperature of 50°C and a broad optimal pH range of 6–8. These cellulases also had high thermotolerance as evidenced by retaining more than 50% activity at 100°C after 1 h. In summary, this is the first study to show that the genus *Brevibacillus* may have strains that can degrade cellulose.

Keywords Cellulase · *Brevibacillus* · Cellulose · Cellulosome

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Introduction

Lignocellulosic materials have been regarded as potential resources for biofuel production. Microbial conversion of cellulose, the major component of plant cell walls, to simple sugars or bioethanol has received intensive attention in the past decades [3]. Certain microbes are categorized as cellulolytic due to their capability of growing on cellulose as sole carbon and energy sources and secreting cellulases [24]. Three kinds of cellulases are required to carry out cellulose hydrolysis: (1) endoglucanases (EC 3.2.1.4), (2) exoglucanases or cellobiohydrolases (EC 3.2.1.91), and (3) β-glucosidases (EC 3.2.1.21) [19]. To hydrolyze cellulose, endoglucanases locate surface sites randomly along the cellodextrin and insert a water molecule in the intramolecular β-1,4-glucosidic bonds, creating a new reducing and non-reducing chain end pair; exoglucanases processively cleave cellulose chains at the ends to release cellobiose, and β-glucosidases convert cellobiose to glucose and thus relieve the system from end-product inhibition [12]. However, the division into

endo- and exoglucanases in many cases is not absolute and may have the risk of over-simplification [2, 10, 11, 14, 29, 30]. A processive endoglucanase in *Thermobifida fusca* E4 has been documented [14], and it has been suggested that exoglucanases could exhibit some endoglucanase activity due to temporary conformational structural changes [33, 38].

Depending on the organization and structure of cellulases, three microbial strategies have been proposed by Wilson for plant cell wall degradation [36]. In terms of anaerobic bacteria, such as the most studied *Clostridium thermocellum* and *C. cellulovorans*, cellulosome, a macromolecular machine (multienzyme complex) is produced for cellulose hydrolysis in an organized, concerted, and synergistic way [3–5, 19, 27]. The components of the multienzyme complex are strongly bound to each other through dockerin: a duplicated, non-catalytic segment of 22 amino acid residues found to be conserved in all enzymes located in the cellulosome [31]. This dockerin module binds specifically to the cohesin modules or scaffoldin, located in a noncatalytic cellulosome component. The catalytic components themselves are complex proteins consisting of catalytic and non-catalytic modules [24]. With regard to aerobic cellulose-degrading bacteria and fungi, a mixture of non-complexed cellulases is secreted to depolymerize cellulose. Each individual cellulase contains a carbohydrate-binding module (CBM) joined by a flexible linker peptide to the catalytic domain (CD). The third strategy was recently proposed to explain the cellulose-degrading behavior of two newly sequenced bacteria: aerobic *Cytophaga hutchinsonii* and anaerobic *Fibrobacter succinogenes* [37]. The genome sequences of these two bacteria revealed that: (1) they do not have any genes encoding any known processive cellulases; (2) no genes in *C. hutchinsonii* were found to code for CBM as other aerobic cellulolytic bacteria do; (3) no genes were identified to encode dockerin and scaffoldin in *F. succinogenes* as other anaerobic cellulose degraders have. Thus, the proposed third mechanism of cellulose hydrolysis by these two bacteria is that the outer membrane proteins are able to bind and transport individual cellulose molecules into the periplasmic space where they are degraded by endoglucanases [37].

Despite extensive research that has been devoted to the field of cellulose utilization, the survey of the literature revealed as few as 75 validly described bacterial species that can grow on crystalline cellulose and use it as a sole carbon and energy source [24, 39]. This study describes a potentially new thermophilic cellulose-degrader that was originally isolated from swine waste and characterized as a *Brevibacillus* strain.

Cellulose degradation under aerobic and thermophilic conditions offers many advantages, such as: (1) aerobic bacteria produce high cell yields that lead to high enzyme production [19]; (2) thermophiles are commonly believed to be robust microorganisms that contain stable enzymes [3]; (3) potential contamination can be easily prevented due to high growth temperatures [3, 26]; (4) the cooling problems for pretreated biomass can be simplified [3]; (5) the products from aerobic cellulose degradation are simple sugars that have the versatility and flexibility to be transformed to many value-added commodities including, but not limited to, ethanol, butanol, hydrogen, and amino acids [4]. Study of the new isolate not only increases the knowledge on diversity of bacteria capable of utilizing cellulose, but also helps to provide insight into the mechanisms of cellulose conversion by aerobes.

Materials and methods

Materials and chemicals

Sigmacell cellulose type 50 was purchased from Sigma Aldrich (St Louis, MO). Carboxymethylcellulose (CMC) was bought from Spectrum Chemical MFG Corp (Gardena, CA). Yeast extract and tryptone were purchased from Difco laboratories (Sparks, MD). All other chemicals used in this study were of the highest grade possible from Fisher Scientific (Pittsburgh, PA) if not noted specifically.

Source of bacteria

Several years ago, a project existed to use an aerobic and thermophilic process to degrade swine waste from a swine finishing building at Southern Illinois University. After extensive laboratory studies, a pilot plant of 3.8 m^3 was built to run for several years at 57°C. Lyophilized samples from this pilot plant served as the original bacterial source for this study.

Isolation of thermophilic cellulose degrader

To 50 ml 0.85% sterile NaCl solution, 0.5 g bacterial source sample was added. The mixture was homogenized in a shaker at 150 rpm for 1 h at 57°C; 10 ml of the mixture was inoculated into 90 ml medium containing (per liter): 2.0 g $CaCO_3$, 1.0 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, and 0.5 g NaCl [1]. After the inoculated culture was shaken at 57°C for 3 days, 100 µl of sample was spread onto a cellulose agar plate (per liter): 20.0 g agar, 10.0 g cellulose, 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 $CaCl_2$ [1]. Without adding agar and cellulose, this medium was referred to as cellulolytic medium and was used throughout this study.

After 3 days, light orange colored colonies appeared on the plate. One colony was further streaked onto cellulose agar plates for purification. Overnight colony growth was observed following each transfer. After four plate transfers, cells were added to Luria Bertani (LB) medium (per liter): 10 g yeast extract, 5 g tryptone, and 10 g NaCl. Aliquots of the 12-h grown LB culture were used: (1) for DNA extraction, (2) to inoculate cellulolytic medium spiked with cellulose, CMC, cellobiose, and glucose for detecting cellulase activities, and (3) to inoculate cellulolytic medium supplemented with different carbohydrates for substrate utilization experiment as described in details below.

16S rDNA gene sequencing and phylogenetic analysis

A total of 1.8 ml of the LB culture was centrifuged ($10{,}000\times g$) to obtain the cell pellet for DNA extraction using UltraClean Microbial DNA kit (Mo Bio Laboratories, Carlsbad, CA). Polymerase chain reaction (PCR) was performed to amplify a portion of the 16S rDNA using universal primers 8F (5′AGAGTTTGATCCTGGCTCAG) and 1492R (5′ACGGCTACCTTGTTACGACTT). The following PCR parameters were adopted for amplification: an initial denaturation step at 95°C (5 min), followed by 35 cycles of 95°C (1 min), 50°C (1 min), 72°C (1 min), with a final extension at 72°C for 10 min. The amplified PCR product was visualized by running gel electrophoresis. The 16S rDNA band was cut and purified using Gel DNA extraction Kit (Qiagen, Valencia, CA). The purified DNA sample was sent to Agencourt (Beverly, MA) for sequencing.

Nucleotide sequence

The partial 16S rDNA sequence of the isolate has been submitted to Genbank. The accession number is EU882157.

Substrate utilization

Overnight LB grown culture was used to examine substrate utilization by the isolate. Several substrates, including cellulose, CMC, xylan, xylose, glucose, and cellobiose, were evaluated. Regarding each substrate, a 20% stock solution (10% stock for xylan) was prepared and autoclaved. To a certain volume of cellulolytic medium in a 250-ml flask, 10 ml of the LB culture was added. Different volumes of substrate stock solution were also added to make the final concentration as 10 g/l in a total volume of 100 ml. The optical density (OD) was monitored daily at 600 nm. With regard to the flask with cellulose, the flask was allowed to settle for approximately 15 min before the suspension was taken for measurement considering the insoluble characteristic of cellulose.

Cellulase activity measurements

Cellulase activities were measured for cells grown in 100 ml cellulolytic medium supplemented with either cellulose, CMC, cellulose plus cellobiose, or cellulose plus glucose in 250-ml Erlenmeyer flasks on a rotary shaker at 150 rpm at 57°C. Cells were also grown in PTYE medium containing (per liter): 5 g of peptone, 5 g of tryptone, and 5 g of yeast extract with or without the presence of cellulose. The concentration for each substrate was 10 g/l. At different time intervals, 1.5 ml of sample was taken out from each flask and centrifuged at $18{,}000\times g$ for 10 min. The supernatant that was also the crude protein sample was analyzed for a filter paper unit (FPU) based on Ghose's procedure [7].

Determination of optimal temperature and pH for FPU assay

Temperatures between 50 and 100°C at 10°C interval and pH between 5 and 10 at 1 unit interval were evaluated to determine the optimal temperature and pH for FPU assay. Supernatant sample from strain JXL grown with cellobiose was tested. The best temperature and pH were then adopted to perform FPU assay for different crude enzyme solutions.

FPU assay

Briefly, 0.5 ml crude enzyme solution was incubated with a filter paper strip (Whatman Grade 1, 1 × 6 cm) in 1 ml citrate buffer (0.05 M, pH 8.0) at 50°C for 1 h. The amount of reducing sugars produced was determined by 3,5-dinitrosalicylic acid (DNS) reagent, with glucose as a standard. In terms of flasks with the presence of cellobiose or glucose as the co-substrate, 0.05 ml of crude enzymatic solution was used due to the high concentrations of sugars in the solution.

Dose effects from cellobiose and glucose on cellulase induction

In order to determine the inducing effects from cellobiose and glucose on cellulase activities, four different concentrations as 0.1, 0.2, 0.5, and 1.0% for each sugar were evaluated. The experiment was set up in the same way as described above. After 24 h incubation, the crude protein samples were analyzed for FPU activities.

Electron microscopic observations

At late log phase, 10 ml of bacterial cultures grown on cellulose or glucose was processed for electron microscopic

observation. Cultures with cellulose were allowed to sit for 15 min, and the cellulose precipitate was removed using a pipetter. This fraction was referred to as the cellulose residue. The remaining suspension was considered the liquid culture as discussed below.

Regarding scanning electron microscopic (SEM) observation, glutaraldehyde (EM Sciences, Hatfield, PA) fixed cells in original media were rinsed three times in Kellenberger buffer [8] containing 0.1% Bacto-Tryptone (Difco Laboratories) and 0.05% NaCl. Cells suspended in the rinse were allowed to settle onto a poly L-lysine (EM Sciences) coated glass slide in the presence of OsO_4 vapors overnight at room temperature. The slides were subsequently rinsed three times in distilled and deionized water (DDW), dehydrated through 25, 50, 75, and 100% ethanol (10 min each), Critical Point Dried (Tousimis Samdri 690), sputter-coated with 40 nm palladium/gold (Denton Desk II), and viewed in a Hitachi S570 SEM (Pleasanton, CA).

For the purpose of observing extracellular structures, cationized ferritin (CF, EM Sciences) was used based on a procedure published by Lamed [17]. In short, a 1-ml sample of culture grown with cellobiose, glucose, or with no substrates was centrifuged. The cell pellet was washed by 0.9% NaCl (saline) twice. A 0.2 ml solution of CF (1 mg/ml) was applied to the cell pellet for 10 min. The pellet was washed with saline, and the cells were fixed with 5% glutaraldehyde overnight. The fixed cells were dehydrated by a series of graded ethanol solutions and prepared for SEM observation as described above.

For TEM, liquid bacterial cultures were mixed with sufficient glutaraldehyde for a final concentration of 2% and refrigerated (4°C) overnight. These were centrifuged (2,000×*g*, 5 min) with the resulting pellet resuspended in Kellenberger buffer containing 2% glutaraldehyde with 1% NaCl and refrigerated overnight. Following centrifugation, the pellets were successively rinsed in Kellenberger buffer with 1, 0.5, and 0.0% NaCl, then resuspended in Kellenberger buffer containing 1% OsO_4 and 0.1% Bacto-Tryptone with 0.05% NaCl and allowed to sit at room temperature overnight. After centrifugation, pellets were rinsed three times in DDW, enrobed in 3% agarose (Sigma Aldrich, St Louis, MO) and then bulk-stained in 2% uranyl acetate while refrigerated overnight. The stained, enrobed cells were then rinsed again three times in DDW, dehydrated in an ethanol series (as above), dehydrated in propylene oxide (three times at 15 min each), and successively infiltrated (24 h in freezer, −4°C) in 25, 50, 75 and 100% Spurr's resin (EM Sciences) diluted with propylene oxide. Specimens were then embedded in 100% Spurr's in BEEM capsules and polymerized at 60°C for 48 h. Sections (80–100 nm) were obtained on a Leica EM UC6 ultramicrotome using a diamond knife, stained with 2% uranyl acetate followed by lead citrate [22], and viewed in a Hitachi H7650 TEM.

Results

Isolate identification

The partial 16S rDNA sequence was attained with a length of 1,400 bps. The taxonomical assignment of this bacterium is 100% *Brevibacillus* genus in the Paenibacillaceae family within the Bacilli class of the Firmicutes phylum based on the classification provided by RDP database. BLAST search of the sequenced 16S rDNA indicated that this isolate is 99% identical to *Brevibacillus* sp. P258 and K9 (AM749778.1 and AM749792.1) isolated from geothermal soils in New Zealand, 98% identical to a novel feather-degrading strain, *Brevibacillus thermoruber* T1E (DQ452015.1), and some other *Brevibacillus* related to thermophilic condition at different continents. The 16S rDNA sequence is also 94% related to that of *Brevibacillus laterosporus*, a species capable of degrading the cuticles of nematodes [13]. Thus, the genus *Brevibacillus* is composed of strains capable of breaking down a range of unique polymers. A phylogenetic tree illustrating the relationship of strain JXL to other *Brevibacillus* species and a select group of cellulose-degraders is depicted in Fig. 1.

Substrate utilization

Based on colony growth on cellulose plates at different temperatures and pH, a temperature of 57°C and medium pH of 7.0 were found to be optimal for cell growth. Under these conditions, strain JXL could utilize polymers including crystalline cellulose, CMC, and xylan (a component of hemicellulose) (Fig. 2). Due to the suspension nature of the xylan stock solution, the initial OD was 0.4. However, the increase of the culture absorbance could still be observed, which indicated cellular growth. All simple sugars tested were taken up by strain JXL. Growth rates for different sugars were: glucose > cellobiose > xylose. Similar results were obtained from repeated experiments.

Cellulase activity measurement

The temperature effect on FPU assay for crude enzyme samples from cellobiose culture is illustrated in Fig. 3. A temperature of 50°C was found to be optimal for crude cellulase activity, followed by 70 and 80°C. At other temperatures, the FPU was significantly lower. However, it was interesting to see that 57.2% of cellulase activities were retained even at 100°C after 1 h. In terms of buffer pH effect (Fig. 4), pH between 6 and 8 were the optimal ones, and no significant difference was observed among the three. Therefore, 50°C and buffer pH 8.0 were used for FPU assay for different enzyme solutions.

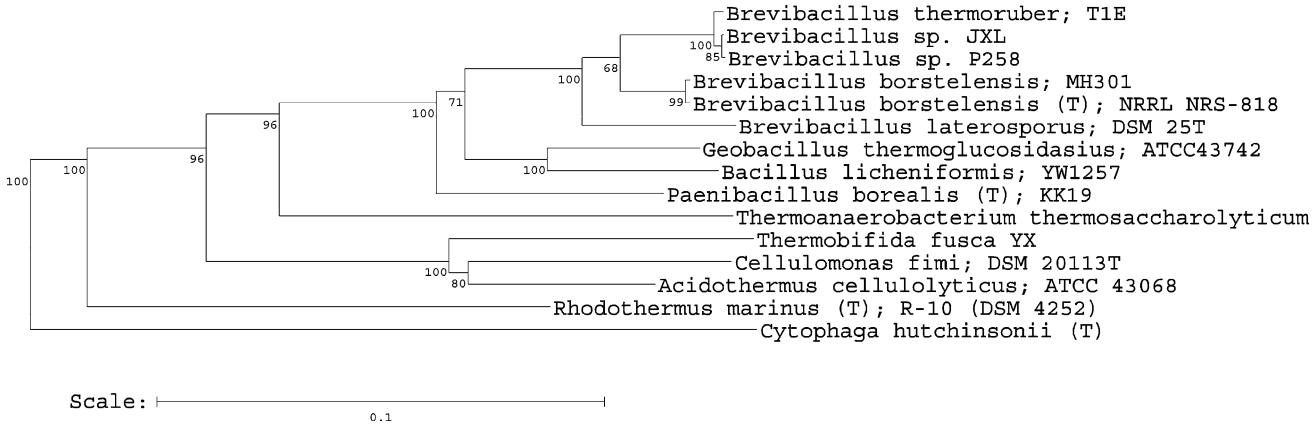


Fig. 1 Phylogenetic tree based on 16S rDNA sequences of *Brevibacilllus* strain JXL, its closest relatives, and select cellulase producing microbes (sequences from the Ribosomal Database Project II). The tree was constructed using the Weighbor tree method (a distance corrected modification of Jukes Cantor). *Numbers* at the nodes indicate bootstrap values based on 100 replicates, while the *scale* indicates the number of changes per nucleotide position

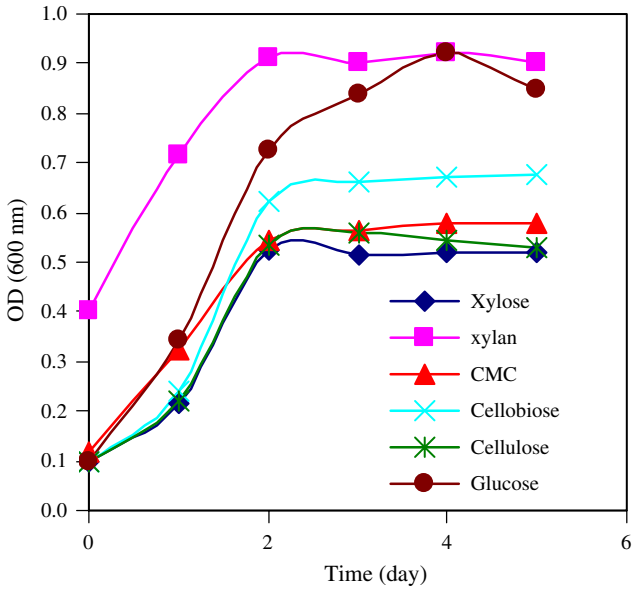


Fig. 2 A typical growth curve of strain JXL grown on different substrates

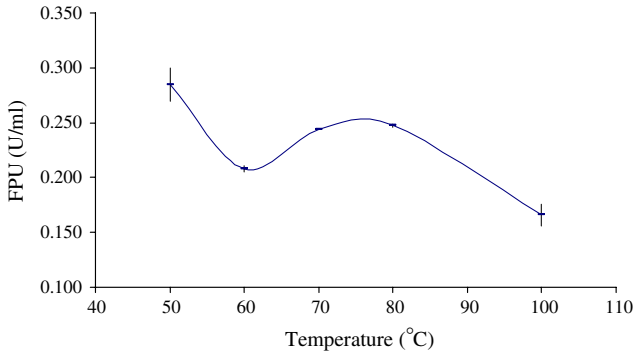


Fig. 3 Temperature effect on FPU assay of crude cellulase mixtures

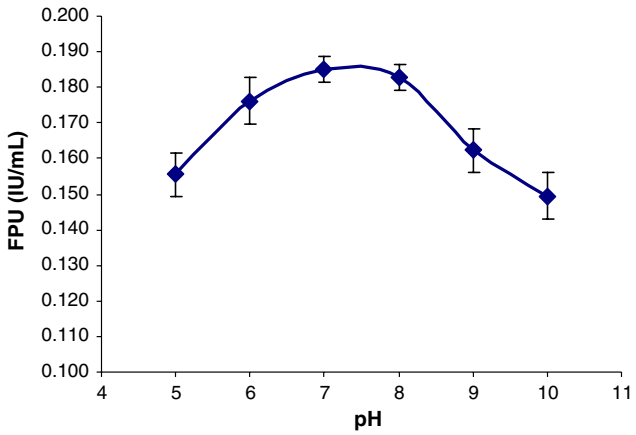


Fig. 4 pH effect on FPU assay of crude cellulase mixtures

Cellulase activities (FPU/ml) of crude protein samples from different substrates are presented in Fig. 5. When the isolate was grown with cellulose or CMC in the cellulolytic medium, an approximate 0.015 FPU/ml was observed from the culture supernatant. This value stayed unchanged during the experimental period of 5 days. Similar enzymatic activities and trend were detected for cells grown with or without cellulose in PTYE medium. No statistically significant difference was demonstrated among these culture samples. However, when cells were grown with cellulose in the cellulolytic medium with the presence of glucose or cellobiose, more than 10–20 times higher enzymatic activities were discovered. During the first 24 h, there was no significant difference between the samples from these two substrates, but after that time, a significant difference was observed. Culture with glucose demonstrated higher cellulase activities compared to those from culture with cellobiose.

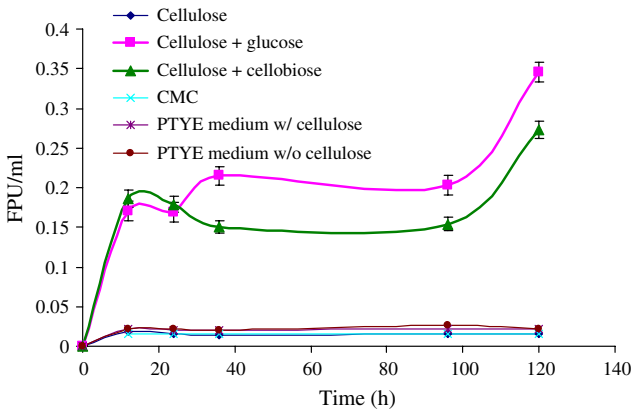


Fig. 5 Cellulase activity measured as filter paper unit (FPU)/ml in the supernatant of strain JXL grown in two different media with cellulose, CMC, cellulose plus cellobiose, cellulose plus glucose, or without cellulose

Dose effects from cellobiose and glucose

As a single substrate, cellobiose and glucose presented inducing effects in the range of 0.1–1.0% (Fig. 6). With the increase of both sugar concentrations, the inducing effects became more prominent. When the concentration was 0.1%, the enzymatic activity was three to four times higher compared to those without the sugars (Fig. 5), whereas the concentration of 0.2 and 0.5% increased the cellulase activities 10 and 20 times, respectively. No significant difference was found between doses of 0.5 and 1.0% for both cellobiose and glucose.

Electron microscopic observations

Electron microscopic observations revealed that strain JXL is a motile (with one flagellum) and spore-forming bacterium. The spores have oval shapes and are smaller than cells (1.8 μm). As shown in Fig. 7a, in cellulose residue, which was the part that was allowed to settle to the bottom of the culture flasks, a fair number of healthy cells and spores were present. In the suspension sample of the same culture, a dark layer of cellulose was adsorbed on cell surface (Fig. 7b), but in glucose-grown cells, this layer was absent (Fig. 7c). Cells grown with glucose showed some protuberant structures on the cell surface, but those structures were not very clear (Fig. 7d).

Aided by CF, the extracellular protuberant structures were more visible. Without the presence of simple sugars, the cells had a rough appearance (Fig. 8a). Growth on cellobiose was accompanied by a denser distribution of the surface protuberances (Fig. 8b), whereas growth on glucose produced a multitude of protuberant structures on the cell surface with larger sizes (Fig. 8c).

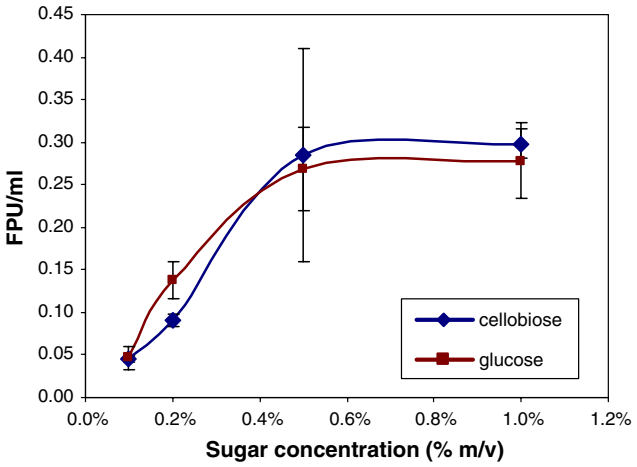


Fig. 6 Dose effects from cellobiose and glucose on cellulase induction

Discussion

To date, few strains of the *Brevibacillus* genus have been shown to be cellulolytic. One *Brevibacillus* sp. strain, M1-5, was indicated to be an anaerobic condition inducer in a cellulose-degrading community, but not a cellulose-degrader itself [15]. Another *Brevibacillus* strain isolated from the gut of the termite *Zootermopsis angusticollis* has indicated CMC, but not cellulose degrading potential when it is grown on filter paper [34]. The closest relatives of strain JXL are strain P258, strain K9, and *Brevibacillus thermoruber*. However, none of these *Brevibacillus* species have been reported to possess cellulose degrading capabilities. Therefore, strain JXL isolated from swine waste may be the first strain of the *Brevibacillus* genus that can be grown on cellulose and used as the sole carbon and energy sources.

Besides growing on cellulose, strain JXL has the ability to utilize other substrates, including CMC, xylan, glucose, cellobiose, and xylose. This broad substrate utilization profile demonstrated the versatility and potential of strain JXL for plant-based material transformation. As uptake of glucose, cellobiose, CMC, and xylan has been reported for other cellulose degraders, the capability of strain JXL for using crystalline cellulose as the only carbon and energy source makes it a true cellulolytic bacterium [24].

In the culture of strain JXL with cellulose, we were interested in knowing where the cells were located. As revealed by SEM and TEM, the cellulose solid residue—the part of culture that was allowed to settle to the bottom of flask—contained a great number of growing cells and spores (Fig. 7a), whereas in the suspension of the culture, cells were also associated with cellulose (Fig. 7b). Therefore, strain JXL showed strong attachment to cellulose.

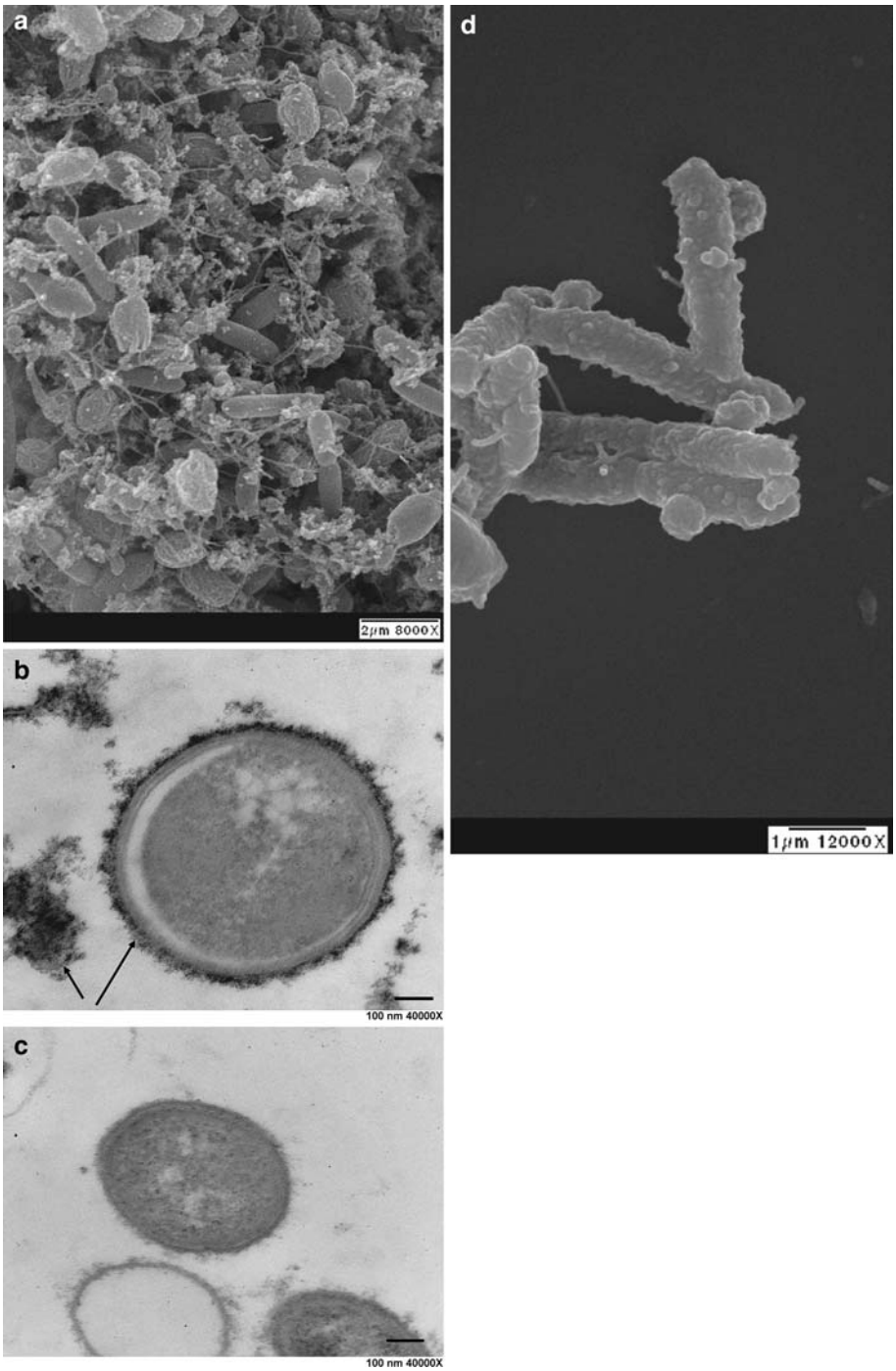
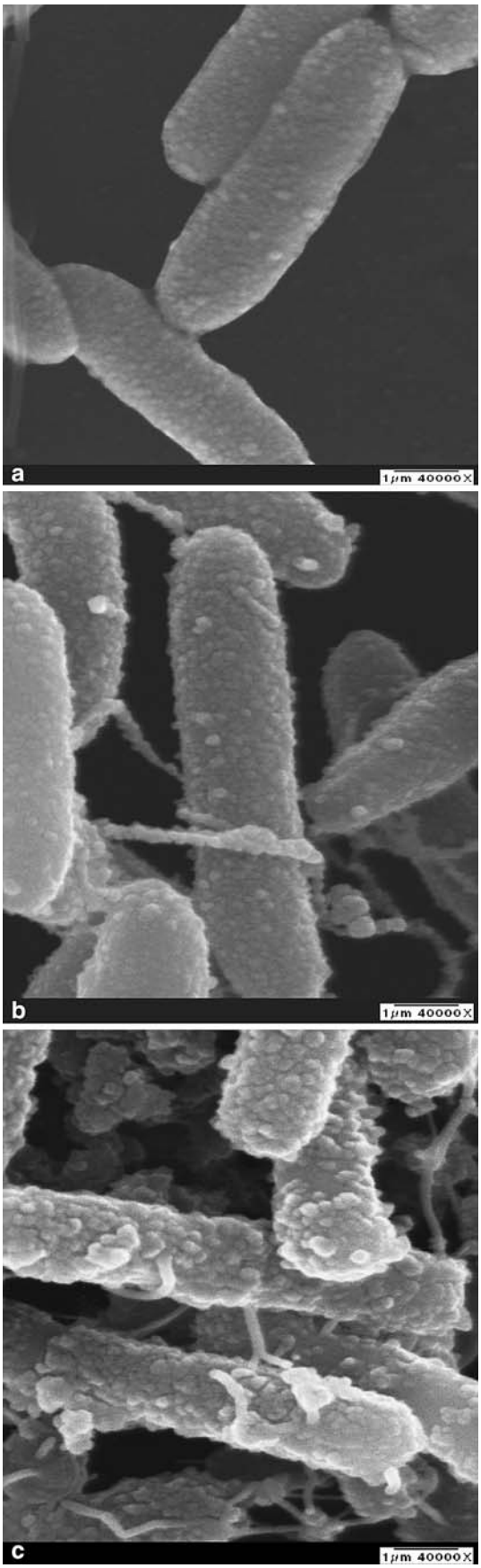


Fig. 7 SEM image of cellulose residue from strain JXL culture with cellulose (**a**), TEM images of suspension sample from cultures with cellulose (**b**), culture with glucose (**c**), and SEM image of culture with glucose (**d**). *Arrows* in (**b**) indicate the cellulose fiber either in culture suspension or adsorbed to cell surface

This property is very important since adherence or adsorption to cellulose is a prerequisite for cellulose hydrolysis [19]. These observations also demonstrated that the OD monitor of the culture suspension could not give an accurate determination of cell growth. However, this was not our concern in this study since we already knew that strain JXL grew on cellulose plates rapidly.

When strain JXL was grown with different substrates except xylan and xylose (for these two, FPU was not conducted), cellulases were released to the culture medium as revealed by the FPU assay. It was interesting to observe that the crude enzymes were even active after they were incubated at 100°C for 1 h. At this denaturing temperature, cellulases induced by cellobiose still retained 57.2% of the activities at their optimal temperature. Hence, the high thermo-tolerance and their relatively broad optimal pH range make them invaluable for future industrial application.



◀ **Fig. 8** SEM images of strain JXL in culture with no sugar addition (**a**), with cellobiose (**b**), and with glucose (**c**). Cells were stained by cationic ferritin first and then observed under SEM

It was found out that the two media used in this study had similar reducing sugar contents (0.17 mg/ml), which was probably from the yeast extract added. Based on the fact that there was no significant difference among the FPU in cultures with either cellulose, CMC, or without cellulose, it was proposed that either the small amount of reducing sugars in the media induced cellulase secretion or a low level of cellulases was produced by strain JXL constitutively. Such a basal level of constitutive cellulases was reported for *Sporotrichum pulverulentum* [6] and *T. reesei* [9]. A similar phenomenon was also discovered for *Cellulomonas* sp. [32] as low levels of endo- and exo-cellulases and very little β-glucosidase were produced from the culture in PTYE medium without cellulose. However, increases of extracellular cellulase activities were not detected during this 5-day study, which was repeated several times, but strain JXL did grow on cellulose and CMC. These facts prompted us to propose that most of the cellulases may be cell-bound and not in free form in culture supernatant. This statement was strongly supported by the SEM images that indicated the presence of extracellular protuberant structures.

Several investigations have concluded that cellulases are inducible enzymes, and different carbon sources have been found to play important roles in affecting the enzymatic levels [16, 18]. Cellobiose and/or glucose have been reported to be good cellulase inducers for many different cellulolytic microbes. For *T. fusca*, cellobiose induces the expression of cellulase genes while glucose acts as a catabolite repressor [35]. Regarding *Bacillus* sp., the presence of 0.2% of cellobiose and glucose increases the rate of endoglucanase biosynthesis [21]. For *Cellulomonas*, cellobiose and xylose at levels from 0.05 to 0.2 g/l serve as inducers for cellulase production [23]. For *T. viride*, cellobiose and glucose appear to be inducers, but not superior ones [20]. In the case of strain JXL, when glucose or cellobiose was used as a co-substrate, much higher cellulase activities were displayed from the culture supernatant compared to those without co-substrate presence. Additionally, the extracellular cellulases were best induced by glucose other than cellobiose.

Similar inducing effects were noted when cellobiose or glucose served as the only substrates as shown in Fig. 6. Compared to FPU obtained from the co-substrate study, higher cellulase activities (0.3 vs. 0.2 FPU/ml) were produced directly from simple sugars during the same experimental period of 24 h. Concentrations of inducers can have dramatic effects on cellulase production. For *Cellulomonas uda*, cellobiose at 0.05 and 0.1 mM has a distinct inducing effect, whereas higher concentrations of 1.0 and 2.0 mM repress endoglucanase formation [25]. Compared to *C. uda*, strain

JXL required much higher inducer concentrations to achieve higher enzymatic activities.

These inducing effects were confirmed by the SEM images. When cells were grown in the cellulolytic medium with no sugar supply, a rough rather than smooth cell surface was visible (Fig. 8a). With the presence of sugars, the extracellular protuberant structures were much denser and were increased in sizes. Between the two sugars tested, glucose produced a significant multitude of such structures (Fig. 8b, c). These protuberant structures are very similar to the cellulosomes described by Lamed for different aerobic and anaerobic cellulolytic bacteria [17].

Few aerobic cellulose-degraders have been proved to bear cellulosomal structures. An aerobic marine bacterium, *Vibrio* sp. MA-138, was presented as possessing dockerin-containing enzymes that serve as the evidence for the existence of cellulosomal structure [28]. A well-studied *T. fusca* has been reported to have extracellular, cellulosomal structures [24]. However, the long-time researcher on this bacterium, Dr. Wilson at Cornell University, has never acknowledged the possible existence of such structures at least to our knowledge. The facultatively aerobic *Cellulomonas* sp. is known to have this kind of extracellular cellulosomal structures when grown on cellulose or cellobiose. These structures, however, are absent when the same cells are grown on glucose [17, 24, 32]. In the case of strain JXL, when it was incubated with glucose, very obvious protuberant structures and higher cellulase activities were detected compared to those with cellobiose. Therefore, there is a strong relationship between extracellular structure and cellulase activities. The cellolusomal-like structures revealed for strain JXL could provide more evidence that cellulosome is strain- and substrate-dependent and may not be a common feature for all cellulolytic bacteria and may not be a unique characteristic just for anaerobic cellulose-degrading bacteria.

In summary, *Brevibacillus* strain JXL is a potentially new gram-positive, aerobic, thermophilic, rod-shaped, motile, and spore-forming cellulose degrader. This is the first study to establish that the genus *Brevibacillus* has the capability to degrade a broad range of carbohydrates, including xylan, CMC, and crystalline cellulose.

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