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Brevibacillus Thermoruber 9X-GLC, Bacteria Isolated from Hot Compost, Producer of a Beta-Glucosidase Resistant to Glucose Inhibition

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Abstract: The thermotolerant strain *Brevibacillus thermoruber* 9X-GLC produces large amounts of β-D-glucosidase and cellobiohydrolase when grown on cellobiose and avicel. 9X-GLC was isolated from hot compost of sugarcane bagasse and filter mud and selected for its high enzymatic activity from a Bacilli collection of 77 isolates from soils and compost. Culture supernatants and ultrafiltered concentrates from shaken flask cultures of 9X-GLC were tested for their β-D-glucosidase activity and stability under different conditions of temperature, pH and glucose concentration. The concentrated supernatant from 9X-GLC cultures on cellobiose as sole carbon source had its highest β-D-glucosidase activity at 55°C; pH= 6.0 and retained 60% of its activity at a glucose concentration of up to 200 mM. The effect of different carbon sources on growth and β-D-glucosidase activity of concentrated supernatants was tested in 48 h shaken flask cultures of 9X-GLC. Glucose, cellodextrin and cellobiose supported high cell densities (>2×10^8 CFU/ml), whereas cellobiose and cellodextrin provided the best substrates for β-D-glucosidase production. The putative gene encoding β-D-glucosidase from 9X-GLC was obtained from PCR amplification and sequenced. The sequence of the putative structural gene of 2085 bp reveals 6 catalytic domains with high (>90%) homology to bacterialglycosidases.

Keywords: β-Glucosidase, Cellulose, Saccharification, Putative Sequence

Introduction

Cellulose is a lineal biopolymer formed by glucose units joined by β-1-4 glycosidic bonds. The structural nature of cellulose makes it insoluble in water and prevents enzyme attack, an effect known as recalcitrance (Himmel *et al*., 2007). Hot compost of lignocellulose by-products of agro-industrial processes have been reported as excellent sources for the isolation of thermotolerant microorganisms useful for the production of oxidoreductases and hydrolases with potential application in the conversion of cellulose to fermentable sugars, biofuels and chemicals (Himmel *et al*., 2007).

Enzymatic degradation of cellulose is an important step in bioethanol production from plant biomass, it requires synergistic action of multiple enzymes, mainly endo-β-1,4glucanase, cellobiohydrolase (exo-glucanase) and β-glucosidase (Fujita *et al*., 2004). Beta-glucosidases are present in bacteria, fungi and plants and show hydrolytic activity on cellobiose and aromatic compounds such as arbutin and silicin present in some plant tissues (Tajima *et al*., 2001; Spiridonov and Wilson, 2001; Park *et al*., 2002; Bogas *et al*., 2007). Enzymatic systems lacking β-glucosidase or those producing it in small amounts show incomplete saccharification of plant cellulose and usually show

product inhibition (Gusakov and Sinitsyn, 1992). Being cellobiose an inhibitor of endo-glucanase, β-glucosidase usually becomes the bottleneck of cellulose saccharification and thus bioethanol production. Accumulation of cellobiose during enzyme hydrolysis can decrease overall cellulose hydrolysis through inhibition when the active site of cellulases is blocked (Sørensen *et al*., 2013), Desirable characteristics of cellulase microbial producers include tolerance to ethanol and thermotolerance when considered as part of a mixed culture for simultaneous saccharification and fermentation processes (Sørensen *et al*., 2013). During enzymatic hydrolysis and saccharification of cellulose, the end-product (glucose) is generally not removed and product inhibition occurs decreasing the reaction rate and cellulose hydrolysis. Most of publications concerning cellulase production have focused on fungal enzymes, nevertheless the use of bacteria for enzyme production has attracted interest, mainly because bacteria show shorter growth period, allowing faster enzyme production. Furthermore bacteria inhabit a wide variety of environments, this makes some strains resistant to extreme conditions. *Bacillus* genus shows a high growth rate and easy adaptability to adverse environments and not excessive nutritional requirements, thus this work aimed to find Beta-glucosidase producers in this microbial group. In this article we report the isolation and primary characterization of a novel thermotolerant and ethanol tolerant bacterium, *Brevibacillusthermoruber* 9X-GLC, as well as its β-D-glucosidase activity. The strain was isolated from sugar cane bagasse hotcompost, it showsβ-D-glucosidase activity highly tolerant to glucose inhibition in concentrated culture supernatants. The strain grows at 55°C and tolerates 5% (v/v) ethanol in the culture medium. 9X-GLC produces a highly glucose tolerant (>200 mM) β-D-glucosidase in submerged fermentations containing eitherglucose, cellobiose, cellodextrins, avicel or xylan, as sole carbon source. The putative structural gene encoding β-D-glucosidase was amplified and sequenced, revealing high homology to bacterial glucosidases.

Materials and Methods

Enrichment Cultures and Isolation of Microorganisms

Soil and compost samples were obtained from different locations in the State of Veracruz, Mexico. For the isolation of microorganisms with β-glucosidase activity, collected samples were suspended in liquid Bacillus Mineral Medium (BMM), with the following composition (g/l): K2HPO4 (0.5); NH4NO3 (0.5); MgSO4 (0.2); and either cellobiose or cellodextrins (0.1) as sole carbon source (De la Cerna, 2011). To obtain spore forming bacilli, soil or compost suspensions were incubated at 80°C during 48 h. Aliquots from the enrichment cultures were then diluted and inoculated on agar plates containing the same mineral medium. Plates were incubated at 50°C for 24 to 72 h. Prevalent colonial morphotypes were isolated and purified. Pure cultures of fast growing gram-positive rods were subcultured and maintained at -70°C or freeze-dried for further use.

Preparation of Enzyme Extracts

Bacterial isolates were cultivated at 50°C during 48 h In Liquid Production Medium (LPM), containing (g/l): K_2HPO_4 (0.1), $MgSO_4$ (0.05), yeast extract (1.0), peptone (1.0), cellobiose (10.0). Each culture was propagated in 250 mL^{-1} Erlenmeyer flasks, containing LPM (50 mL^{-1}) using a rotary shaker at a fixed rate of 150 rpm. After propagation, the culture was centrifuged at 5000 rpm for 15 min at room temperature and the supernatant was ultrafiltered through a 100 kDa membrane (Millipore, USA). Filtrate was then concentrated by subsequent ultrafiltration through a 1 kDa membrane (Millipore, USA) The active ultrafiltration extracts were sterilized by microfiltration through 22 μm filters (Millipore, USA).

Production of Cellodextrins

The method for the production of cellodextrins was based on De la Cerna (2011) and Eveleigh, D.E. (personal communication, 2009). Microcristalline cellulose (Avicel) (20 g) were suspended in distilled water (50 mL^{-1}) and hydrolyzed by slow addition of 100 mL^{-1}HCl followed by 20 mL^{-1} H_2SO_4. The mixture was then heated at 100°C for 10 min and cooled down at room temperature. The pH was adjusted to 7.0 with KOH (40% w/v) and the mixture diluted with distilled water to a final volume of 200 mL^{-1}. The mixture was then transferred to a 1 L Erlenmeyer flask. For the final step of cellulose hydrolysis, 2 mL^{-1} of endoglucanase (Celluclast 1.5L, Sigma Aldrich, USA), were added to the prehydrolyzed cellulose preparation. The reaction was carried at 45°C for 3 h under agitation in a rotary shaker at a fixed rate of 150 rpm. The mixture was then centrifuged to separate insoluble residual cellulose and supernatant was cooled down to reach 4°C and treated with cold ethyl alcohol (96% v/v from a local supplier). Addition of ethanol (100 mL^{-1} at -20°C) was used to obtain a cloudy suspension containing colloidal cellodextrins. The solution was ultrafiltered through a 1 kDa membrane (Millipore, USA) in order to obtain a concentrated retentate (approximately 4.8 g) of a white mass with mainly soluble cellodextrins. The concentrated preparation was washed twice with 25 mL^{-1} of cold ethyl alcohol (96 % v/v) and the insoluble residue was air-dried. The final dried preparation contained 4.2 g of pure, water-soluble cellodextrins.

β-glucosidase Activity Assay

β-glucosidase activity was assayed through a method based on that previously described (Cai *et al*., 1998) by measuring the presence of ρ-nitrophenol liberated by enzyme hydrolysis of ρ-nitrophenylβ-D-glucopyranoside (Sigma Aldrich). Reaction media consisted of 200 µL phosphate buffer 0.1 M pH 6; 700 µL ρ-nitrophenylβ-D-glucopyranoside 10 mM (Sigma Aldrich, USA) and 100 µL of enzyme extracts. The enzymatic reaction was performed at 45°C for 30 min and stopped by the addition of 200 µL of sodium carbonate 1 M. Absorbance was measured at 405 nm using a spectrophotometer (Hatch DR 5000, USA) and the enzyme activity was expressed as β-glucosidase Units, where one Unit was defined as the amount of enzyme needed to produce 1 µmol of ρ-nitrophenol per minute.

Preparation of 9X-GLC cell lysate

After cultivation of 9X-GLC at 45°C during 48 h, 100 m L^{-1} of broth from LPM were centrifuged at 4835 g for 20 min at 4°C. The whole cell pellet was resuspended in 1 m L^{-1} of deionized water. To the cell suspension, glass beads (106 µm diameter, Sigma Aldrich, USA) were added (0.1 g) along with 0.05 g of glass microbeads (3.3 mm diameter). The mixture was incubated at -20°C for 1 h. After thawing, the mixture was vigorously shaken with a vortex for 5 min and then incubated again at -20°C for 5 min. This freeze-shake procedure was repeated two more times. After final thawing, 100 µL solution containing lysozyme (0.5 mg mL^{-1}) were added and the mixture was gently shaken for 50 min at room temperature followed by vortexing for 2 min. Mixture was then centrifuged for 10 min at 4835 g at 4°C. The supernatant was recovered and PMSF added to a final concentration of 1 mM. The lysis supernatant was then ultrafiltered through a 0.22 µm filter and placed in a phosphate buffer (pH 6.0; final concentration 0.1 M).

Identification of 9X-GLC Strain

Genomic DNA from isolate 9X-GLC (DNA extraction kit from Zymoresearch, USA) was used as a template to amplify 16S rDNA sequence using the primers CU-01P46F 3´and CU-031-P1540R, described by Edwards *et al*. (1989). A PCR reaction was performed in a mixture containing 0.2 µM of each primer, 0.5 µg of genomic DNA, 200 µM of dNTPs and 0.05 U of DNA Polymerase (HotStarTaq Plus DNA Polymerase, QIAGEN, USA) with 5 µl of 1x PCR buffer. The reaction mixture was incubated for 25 cycles as follows: 95°C/5 min, 94°C/30, 60°C/30 sec, 72°C/1 min and a final extension time of 1 min at 72°C. The amplification PCR product was purified using the QIAquick Gel Extraction Kit (QIAGEN, USA) and sequenced. The sequence obtained was analyzed by alignment with sequences from the NCBI database. The sequence was also used to build a phylogenetic tree using the program Phylogeny.fr (Dereeper *et al*., 2008). For the construction of the phylogenetic tree with 16S rRNA, a group of sequences from related *Bacillus* and *Brevibacillus* species were used, which are identified with their accession numbers (AB006942.1, JX517229.1, KP004889.1, NR_074984.1, NR_116017.1, KC352741.1, DQ923480.1, AY294325.1, KJ722521.1, KJ123715.1, LN849704.1, EU239110.1).

Results

Isolation of Microorganisms with β-Glucosidase Activity

Spore-forming thermotolerant bacilli strains were isolated from 17 compost and soil samples. The isolates obtained were cultured at either 50 or 25°C. Only 4.2% of all isolates grew at 50°C and 25°C, while 95.8% grew exclusively at 25°C. Cellodextrins were used as carbon source only by 20% of all isolates, the remaining isolates could only grow on cellobiose. All bacillus isolates were tested for their ability to grow on different carbon sources using BMM. Thirteen isolates were selected for their ability to grow on both cellobiose and cellodextrins. Isolate 9X-GLC was able to grow on agar plates and liquid cultures using cellodextrins or cellobiose as a sole carbon source.

Biochemical Characterization of Extracts

Thirteen enzyme extracts from selected isolates were also evaluated with respect to their β-glucosidase activity at different pH and temperatures. Table 1 presents results on the optimal temperature for β-glucosidase activity in UF concentrates of culture supernatants for different bacillus strains. Most extracts showed the highest enzyme activity at 45°C. But extracts from 4 particular isolates showed optimal enzyme activity at temperatures higher than 50°C: 10C, 11C, 2AIC and 9X-GLC. Crude UF concentrates of culture supernatants were also tested for thermal stability and glucose inhibition of β-glucosidase activity.

Figure 1 shows the results of thermal stability of β-glucosidase of 6 selected concentrated extracts, incubated for 4 h at 50°C. Extracts from isolates 2AIC, 3AIC and 9X-GLC maintain 70-80% of the initial enzyme activity, while all other extracts showed only 50-60% of the initial activity. The strain 9X-GLC showed high thermal stability, since it retained 75% of β-glucosidase activity after 4 h of incubation at 50°C.

The effect of glucose (0 to 200 mM) on β-glucosidase activity from selected bacillus culture extracts is presented in Fig. 2. The enzyme extracts from 9X-GLC retain 57% of the initial enzyme activity after incubation for 30 min with glucose, at a concentration of 200 mM.

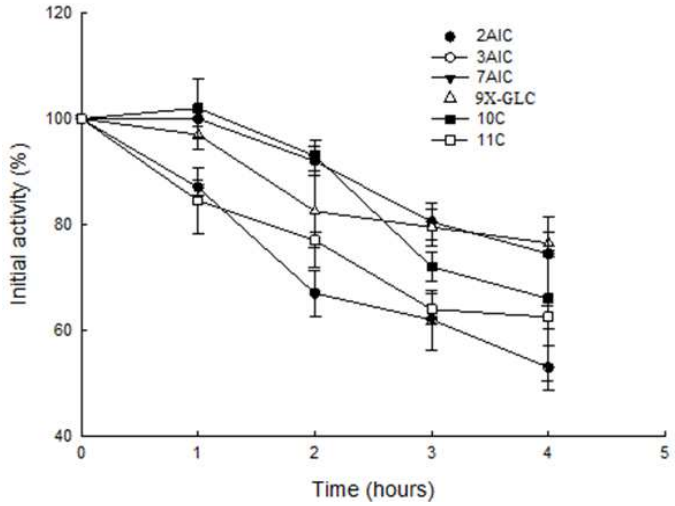

Fig. 1. Thermal stability of β-glucosidase in culture extracts of selected Bacillus cultures, isolated from soil and compost

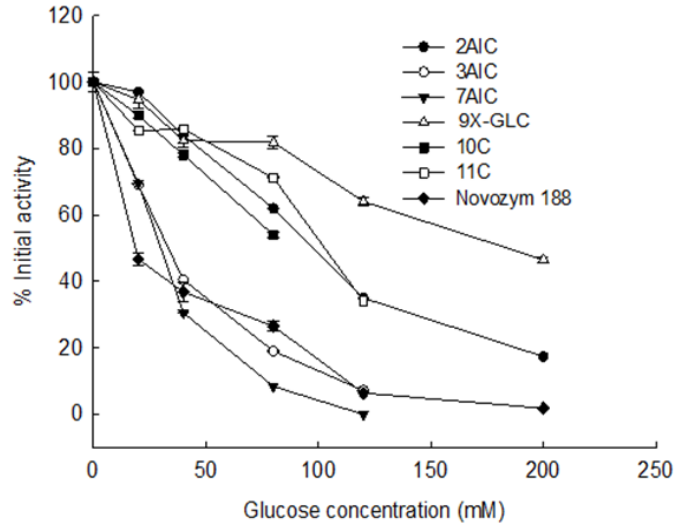

Fig. 2. Effect of glucose concentration on β-glucosidase activity (30 min at 45°C), of selected culture extracts from bacillus isolates

Table 1. Optimal temperature for glycosidase activity in extracts from selected isolates

Isolate	Optimal temperature activity (°C)	Maximal enzyme activity (mU)
1AIC	45	39.21
2AIC	65	44.33
3AIC	50	39.01
4AIC	45	29.26
5AIC	45	32.45
6AIC	50	32.49
7AIC	45	31.10
8AIC	45	32.30
9X-GLC	55	76.28
10C	60	37.30
11C	55	58.20
12C	45	46.20
13C	45	26.60

Table 2. Different carbon sources used by *Brevibacillusthermoruber* 9X-GLC

Carbonsource	CFU X 10^6
Xylan	180±14.1
Glucose	455±35.3
CMC	190±14.1
Pectin	17.5±3.5
Avicel	45±7
Cellobiose	305±7
Cellodextrins	230±14.1
Xylose	215±35.3

A commercial enzyme preparation, Novozym 188 (Sigma-Aldrich), with higher β-glucosidase activity than the produced by the selected isolate (ca. 500 times), showed a very high thermal stability (almost 68% of the initial activity at 50°C, after incubation for 4

h), but a sharp drop (98%) of activity, when incubated in the presence of 200 mM glucose (3.6% w/v). Figure 3 shows enzyme activity of concentrated culture extracts from 9X-GLC strain after cultivation in media containing different carbon sources. Extracts obtained from 9X-GLC cultures containing either cellobiose or cellodextrins showed the highest β-glucosidase activities.

The effect of the carbon source on the ability of 9X-GLC strain to grow in LPM at 45°C is presented in Table 2. The results show that glucose, cellobiose and cellodextrins were the best substrates for 9X-GLC growth, while pectin showed the lowest viable cell yield after 48 h incubation.

Thirteen selected bacillus strains were tested for their ability to grow in presence of ethanol (5% v/v) as this is an important issue for simultaneous saccharification and fermentation processes. From these isolates, only 5 showed growth at a detectable rate Fig. 4. The strain 9X-GLC showed a clear ability to grow in the presence of ethanol.

Presence of Enzyme Activity

The β-glucosidase activity from cell lysates of 9X-GLC is higher than the activity present in supernatants of the same culture (Fig. 5A). This was found after comparison of supernatant and disrupted cells extract. It is showed in Fig. 5B that 45°C was the optimal temperature for β-glucosidase production during 9X-GLC cultivation in liquid media.

Molecular Identification of 9X-GLC

The sequence obtained from 16S rDNA amplification was used to construct a phylogenetic tree (Fig. 6). This phylogenetic tree and the homology percentage of the sequence identify this isolate as *Brevibacillusthermoruber*.

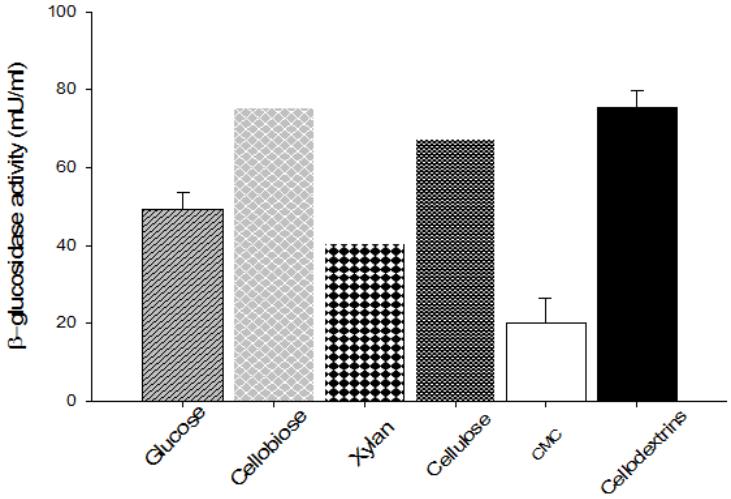

Fig. 3. Effect of the carbon source, on the β-glucosidase activity of concentrated extracts obtained from LPM cultures of 9X-GL after 48 h at 45°C

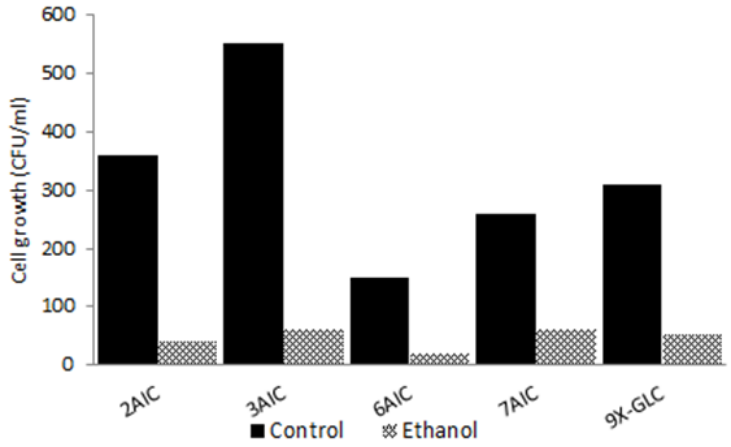

Fig. 4. Growth of selected Bacillus strains in LPM media containing ethanol at 5% (v/v), after 48 h at 45ºC

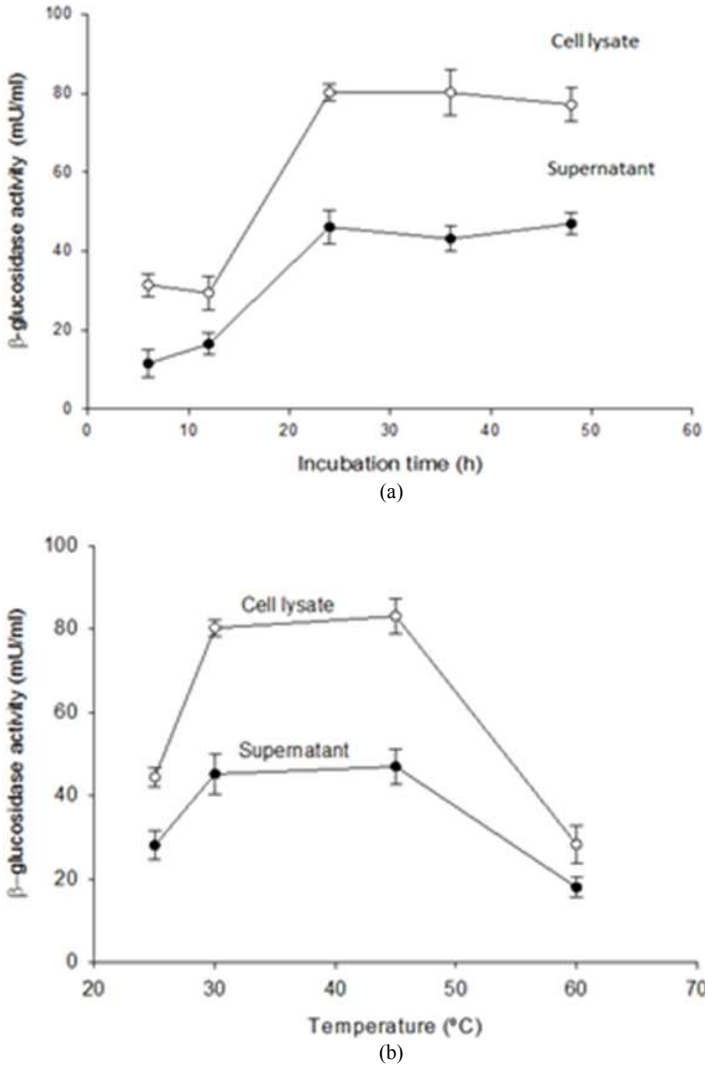

Fig. 5. β-glucosidase activity in supernatant and cell lysate fractions from cellobiose containing LPM cultures of 9X-GLC. (A) Time course of enzyme production at 45°C. (B) Effect of cultivation temperature on enzyme activity produced at 48 h

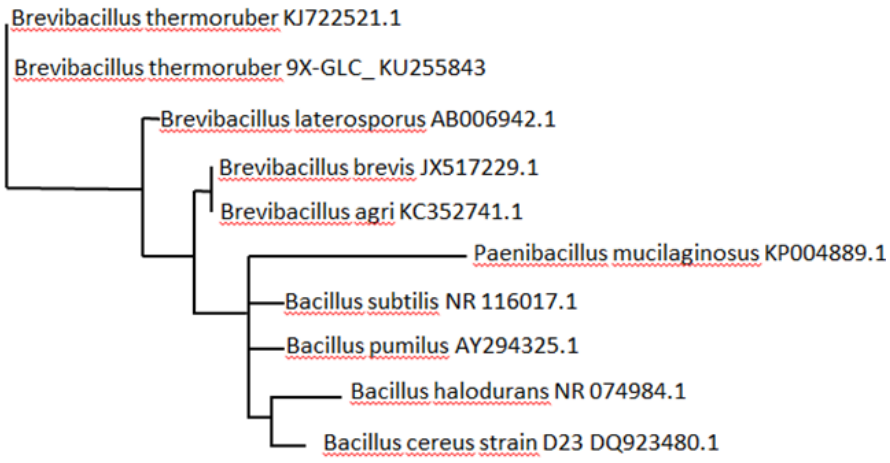

Fig. 6. Phylogenetic relationship of *Brevibacillusthermoruber* 9MX_GLC with related species

It is observed that *Brevibacillusthermoruber*, access number KJ722521.1 from NCBI has the maximal identity percentage to the selected study strain *Brevibacillusthermoruber* KU255843 with 97% identity. Furthermore, the identification of the strain was also confirmed by a professional molecular identification service (Accugenix, Delaware, USA) (not presented data).

Discussion

When cultured in LPM containing cellodextrins, a high cellobiohydrolase activity was measured in the ultrafiltered culture supernatant (<100 kDa, > 1 kDa) of 9X-GLC, obtained from 48 h cultures at 45°C. The enzyme activity was 55 mU mL^{-1} at 50°C using ρ-nitrophenyl β-D-cellobioside as substrate. According to most authors (den Haan *et al*., 2013; Zoglowek *et al*., 2015), this enzyme is responsible for glucooligosaccharides hydrolysis, producing both glucose and cellobiose as main reaction products. This is to our knowledge the first reported *Bacillus* species capable of simultaneous catabolism of cellobiose and cellodextrins with quantifiableenzyme activity of β-glucosidase and cellobiohydrolase. The ability to catabolize both cellobiose and cellodextrins is relatively unusual among microorganisms. Some microbial isolates have been reported to use both cellobiose and cellodextrins as carbon sources. Yeast isolates such as *Candida wickerhamii, Candida lusitaniae* and *Dekkera intermedia* (Freer and Detroy, 1982; Kilian *et al*., 1983; Blondin *et al*., 1982) were found to successfully hydrolyze cellobiose through β-glucosidase, but only *C. wickerhami* could metabolize both cellobiose and cello oligosaccharides. Anaerobic bacteria have also been reported to catabolize cellobiose and cellodextrins. Classic examples are: *Bifidobacterium breve* and *Bacteroidespolypragmatus* (Pokusaeva *et al*., 2011; Mackenzie *et al*., 1986), both strains capable of hydrolysis and catabolism of these oligosaccharides.

The optimal temperature for enzyme activity of 9X-GLC and thermal stability were found high among evaluated isolates. Results reported in a purified β-glucosidase produced by *Bacillus subtilis* (Argungu *et al*., 2014), showed optimal temperature at 60°C and pH 7.0. On the other hand, during the characterization of β-glucosidase from an isolated *Bacillus halodurans* strain, the optimal conditions were 45°C and pH 8.0 (Naz *et al*., 2010). Enzyme activity and stability at high temperatures (>50°C) are important features in cellulases used in bioethanol production, especially in SSF processes (Singhania *et al*., 2013).

Inhibition of β-glucosidase activity by glucose is a common problem during hemicellulose saccharification and also a keystone of bioethanol production by the use of saccharifying enzymes (Saha and Bothast, 1996). During a study with aryl-β-glucosidase from *Trichoderma* spp, enzyme activity was completely inhibited at 1% (w/v) glucose; while the characterized enzyme produced by *Microsporabispora* (Waldron *et al*., 1986) was inhibited by 35% in the presence of glucose at a concentration of 10% (w/v). Strain 9X-GLC was selected from all microbial isolates, because of its high resistance to glucose inhibition, as well as its high thermal stability.

Carbon sources tested for β-glucosidase induction in 9X-GLC showed that cellobiose was the best inducer of enzyme activity, these results are similar to those reported in bacteria isolated from soil (Busto *et al*., 1995) where cellobiose was notably a better inducer of β-glucosidase activity than carboxymethylcellulose. Being cellobiose and cellodextrins the natural substrates for β-glucosidase in this microorganism, it seems clear these substrates promote the enzyme expression.

β-glucosidase activity from cell lysates of 9X-GLC were higher than the activity present in supernatants of the same cultures. These results are consistent to other report (Kim *et al*., 2012), which suggests that β-glucosidases are mainly bound to bacterial cells in *Bacillus* and related genera. In the referred study with bacillar isolates from agricultural environments, it was found that all β-glucosidase activity in *Bacillus subtilis* strains was located only in the whole cell pellet, while no activity was detected in culture supernatants. Furthermore, in a work with *Bacillus licheniformis* (Dhillon *et al*., 1985), the strain could grow in a medium with cellobiose as the sole carbon source, but it was not possible to detect β-glucosidase activity neither in the cell fractions, nor in the culture supernatants The authors attributed the ability to catabolize cellobiose to the initial reaction of cellobiose phosphorylase.

During a work not presented in this study, a gene related to β- glucosidase activity was sequenced and deposited in the NCBI GenBank database under accession number KU25584. The sequence was annotated using the bioinformatics tool RAST (Aziz *et al*., 2008). To obtain the sequence, oligonucleotides were designed from a gene present in the genome with accession number GCA_000454065.1 from *Brevibacillusthermoruber* 423 (Yildiz *et al*., 2013), since it is the identity for 9X-GLC according to mentioned results. By the use of the alignment tool BLAST from NCBI, a gene belonging to the superfamily 3 of glycosidases was identified. The gene is described as β-hexosaminidase (EC 3.2.1.52) and contains 2118 base pairs. This group of enzymes is widely present in bacteria and yeast and the enzymes present in this family are β-glucosidase, Beta-hexosaminidase or chitobiase.

The partial sequence obtained consisted of 709 base pairs and the local alignment of this sequence gave identity to the family 3 of glycosidases. From the genome walking amplification, 4 sequences were obtained and subsequently sequenced via the Illumina

technology. A total of 314,356 reads was obtained. These sequences and the previous 709 bp sequence were assembled using Genious ™ version 8.1.2 (Kearse *et al*., 2012) to get an assembled 3405 base pair sequence, from this sequence an ORF containing 2085 base pairs was identified which corresponds to a member of the family 3 of glycosidases. Its alignment and comparison in databases showed strong homology to catalytic domains present in others glycosidases, mainly in Beta-hexosaminidases and β-glucosidase present in *Bacillus* and related genera.

From a Basic Local Alignment Search and Conserved Domains Finder Tool (Marchler-Bauer *et al*., 2009; Marchler-Bauer and Bryant, 2004), BglX domain is described as a sequence belonging to a β-glucosidase or a related glycosidase. As it is confirmed by some authors (Painbeni *et al*., 1992; Busto *et al*., 1995; Kim *et al*., 2012) glycosidases such as β-glucosidase are enzymes with variable activity, acting on substrates like cellobiose, arbutin, salicilin, esculin, chitin aglycones or non-reducing ends of cellodextrins.

To analyze if the active site profile of this protein may give information about the family of β-glucosidase, it was constructed a structural model for the protein sequence with the I-Tasser server (Zhang, 2008; Roy *et al*., 2010; Yang *et al*., 2015), the best model presented a good score of C (-1.66) with a TM score of 0.51±0.15. The structural model correlate to the glycoside hydrolases of group 3, in particular to the subfamily of β-glucosidases of two domains: one N-terminal domain the $(\beta/\alpha)_8$ barrel fold (TIM-barrel) and C-terminal domain $\alpha\beta\alpha$ sandwich fold, which is structural homolog to the structure of N-acetylglucosaminidase of *Bacillus subtilis* (PDB id: 3bmx): The best structural model constructed by I-tasser presented the best structural alignment (TM-score of 0.791) with the β-glucosidase of *Bacillus subtilis.*

A characteristic of the binding site of the two domain N-acetylglucosaminidase analyzed is the presence of the Asp-His catalytic dyad, which suggests a unique glycoside hydrolysis mechanism similar to the catalytic triad of serine proteases.

When both structures were aligned the catalytic dyad residues His234 and Asp318 of N-acetylglucosaminidaseof *Bacillus subtilis* match structurally with the position of the residues His and Asp that form the catalytic dyad present in our model. Also it is present a homolog residue in *Bacillus subtilis,* the Asp at position 232 that is in hydrogen bond distance for the His and it is required for the mechanism acid/base.

In a study (Mayer *et al*., 2006) two enzymes present in *Cellulomonasfimi* were characterized, aβ-N-acetylhexosaminidase and a β-N-acetylglucosaminidase/β-glucosidase. The β-N-acetylglucosaminidase found showed β-glucosidase activity and belongs to the glycosidase family 3 as well as the protein related to gene sequenced in our work. A glycosyl hydrolase belonging to family 3 (Choi *et al*., 2009) was identified as β-N-acetylhexosaminidase, it showed specificity against β-linked N-acetyl glucosamine sugars and showed activity on pNPGlc, the substrate commonly used for β-glucosidase assay. Other reports have demonstrated the broad activity of glycosyl hydrolases on different substrates (Painbeni *et al*., 1992; Busto *et al*., 1995; Kim *et al*., 2012). Despite the showed capability of 9X-GLC to grow on media with cellobiose or cellodextrins, as well as the measured β-glucosidase activity of the strain and the characterization of the sequenced generepresentindicia suggesting that the gene described here encodes an enzyme with β-N-acetylglucosaminidase and β-glucosidase. Further research is needed to fully describe the specificity of this enzyme and contribute to the annotation of this group of hydrolases.

Conclusion

A bacillus Gram positive strain was isolated from hot compost, with 16S ribosomal sequence with 97% identity reported for *Brevibacillusthermoruber*, the isolated microorganism is able to grow on media with cellobiose or cellodextrins as only energy source. From a characterization on optimal pH and temperature for activity of β-glucosidase, *Brevibacillusthermoruber* 9X-GLC extract presented its optimal activity at 55°C and pH 6.0, furthermore its resistance to glucose is noticeable. A gene codifying for this enzyme was amplified and sequenced, such enzyme has high identity percentage with reported glycosidases genes like Beta-glucosidases, chitobiases and Beta-hexosaminidase. The enzyme produced by this strain could be used for the production of fuels and chemicals starting from lignocellulosic plant biomass.

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Author's Contributions

Edgar Arturo Téllez-Espino: Worked on biochemical characterization of enzyme activity, molecular identification of bacterial isolate, gene amplification and sequencing as well aswriting of the document.

Sergio Rubén Trejo-Estrada: Designed the study strategy and objectives, worked on reviewing and writing of the manuscript as well asanalysis and interpretation of data.

Carla De la Cerna-Hernández: Developed bacteria isolation and screening, analysis of results and manuscript revision.

Miguel Ángel Plascencia-Espinosa: Supervised and designed the microbial isolation and biochemical screening. Worked on the manuscript reviewing.

María Eugenia Hidalgo-Lara: Advised the team on molecular identification of the selected strain. Supervised amplification of encoding gene of the enzyme andmanuscript reviewing.

Alejandro Sosa-Peinado: Developed in silico prediction of protein structure and function and manuscript reviewing.

Ethics

There are not any ethical issues to declare that could arise after the publication of this manuscript.

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