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Genomic mining of *Geobacillus stearothermophilus* GF16 for xylose production from hemicellulose-rich biomasses using secreted enzymes

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ABSTRACT

The valorization of lignocellulosic biomass, derived from various bio-waste materials, has received considerable attention as a sustainable approach to improve production chains while reducing environmental impact. Microbial enzymes have emerged as key players in the degradation of polysaccharides, offering versatile applications in biotechnology and industry. Among these enzymes, glycoside hydrolases (GHs) play a central role. Xylanases, in particular, are used in a wide range of applications and are essential for the production of xylose, which can be fermented into bioethanol or find use in many other industries. Currently, fungal secretomes dominate as the main reservoir of lignocellulolytic enzymes, but thermophilic microorganisms offer notable advantages in terms of enzyme stability and production efficiency. Here we present the genomic characterization of *Geobacillus stearothermophilus* GF16 to identify genes encoding putative enzymes involved in lignocellulose degradation. Thermostable GHs secreted by *G. stearothermophilus* GF16 were investigated and found to be active on different natural polysaccharides and synthetic substrates, revealing an array of inducible GH activities. In particular, the concentrated secretome possesses significant thermostable xylanase and β-xylosidase activities (5×10^3 U/L and 1.7×10^5 U/L, respectively), highlighting its potential for application in biomass valorization. We assessed the hemicellulose hydrolysis capabilities of various agri-food wastes using the concentrated secretome of the strain cultivated on xylan. An impressive 300-fold increase in xylose release compared to a commercially available cocktail was obtained with the secretome, underscoring the remarkable efficacy of this approach.

Introduction

The development of a society based on bioeconomy relies on an efficient and sustainable use of renewable resources. In this context, biorefineries represent an essential pillar for the development of efficient bioprocesses to produce biofuels and bioproducts from biomass. Therefore, the growing need for green energy sources has brought attention to the valorization of lignocellulosic biomass, particularly the use of spent materials as feedstocks. Renewable residues can originate from household, industrial, and agricultural wastes, thereby yielding value-added products within specific production chains, all the while diminishing environmental impact [1]. Lignocellulosic wastes have high cellulose and hemicellulose content, and efficient biotransformation depends on microorganisms that possess arrays of carbohydrate-active enzymes (CAZymes). While substantial progress has been made in identifying the enzymes employed by biomass-degrading organisms,

Abbreviations: AAs, Auxiliary Activities; ANI, Average Nucleotide Identity; CAZome, Carbohydrate-Active enZymes encoded by the genome of an organism; CAZymes, Carbohydrate-Active enZymes; CBMs, Carbohydrate-Binding Modules; CBP, Consolidated Bioprocesses; CEs, Carbohydrate Esterases; CMC, Carboxymethyl Cellulose; COGs, Cluster of Orthologous Groups; CRISPR, Clustered Reguled Interspaced Short Palindromic Repeats; DNS, 3,5-Dinitrosalicylic Acid; GHs, Glycoside Hydrolases; HPAEC-PAD, High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection; LB, Luria Bertani; NCBI, National Center for Biotechnology Information; PLs, Polysaccharide Lyases; *p*NP, *p*-nitrophenol; *p*NPαAra,, *p*-nitrophenyl-α-l-arabinofuranoside; *p*NPβGal,, *p*-nitrophenyl-β-d-galactopyranoside; *p*NPβGlu,, *p*-nitrophenyl-β-d-glucopyranoside; *p*NPβMan,, *p*-nitrophenyl-β-d-mannopyranoside; *p*NPβXyl,, *p*-nitrophenyl-β-d-xylopyranoside; PULs,, Polysaccharide Utilization Loci; XSEC, Secretome of *G. stearothermophilus* GF16 grown on minimal medium supplemented with 0.1 % xylan.

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there are many important aspects yet to be explored [2,3]. The continued discovery of new microorganisms and their lignocellulosic enzymes is a prominent area of inquiry generating significant interest for addressing our sustainability needs [2,4,5]. The enzymes that degrade holocellulose include glycoside hydrolases (GHs), carbohydrate esterases (CEs), auxiliary activities (AAs), and polysaccharide lyases (PLs), whose action can further be improved by appended non-catalytic carbohydrate-binding modules (CBMs), all classified at the CAZy database (www.cazy.org) [3]. Combinations of these enzyme types play a key role in carbohydrate degradation and are employed also in a variety of different industrial applications. For example, xylanases have been used to bleach pulp and paper, improve the digestibility of animal feeds, clarify fruit juices, and convert plant biomass into valuable feedstocks [6,7]. Also, the production of xylose from lignocellulosic biomass, including agricultural residues, is pivotal for cost-effective biomass valorization, as it can be a significant component of some lignocellulosic residues (up to about 25 % of the polysaccharide content for some specific agri-food wastes) [8–10]. Xylose can be fermented into bioethanol as a sustainable energy source, and also represents a building block for applications in the food, feed, and textile industries [11–13]. Moreover, xylose and xylooligosaccharides are prebiotics, encouraging the growth of beneficial gut bacteria that promote digestive health and inhibit colonization by harmful microorganisms [7,14].

To date, knowledge of cellulolytic and xylanolytic CAZymes is mainly derived from filamentous fungi; many enzymes involved in lignocellulose degradation have been extensively studied from various fungal species, such as *Clonostachys byssicola*, *Postia placenta, Aspergillus niger*, *Phanerochaete chrysosporium*, *Ustilago maydis*, *Fusarium solani*, etc. [15,16]. However, fungi often require more complex culture conditions and media compositions compared to bacteria [17]. Therefore, the optimization of enzyme production may require several days, specific nutrients and pH levels which can add complexity and cost to the industrial process; in addition, the fungal secondary metabolites can interfere with enzyme production or downstream processes [18,19]. In this context, certain species of thermophilic microorganisms and their enzymes (thermozymes) offer several advantages: heat tolerance, high robustness in processes involving thermal pre-treatments, and reduction of cooling costs required by mesophilic microorganisms [20–23]. Moreover, the high stability of thermozymes is an essential feature for bioconversion conditions requiring high solubility of reactants and products, reduced risk of contamination, and improved enzyme accessibility thanks to lowered substrate viscosity [23–27].

Among thermophilic Bacteria and Archaea, those belonging to the genera *Alicyclobacillus*, *Clostridium, Geobacillus*, *Pyrococcus*, *Saccharolobus*, *Thermus*, *Thermobifida* and *Weizmannia* are known to provide industrially relevant thermostable enzymes that are pivotal for enhancing lignocellulosic biomass conversion [28–33]. For example, comprehensive reviews and recent analyses of numerous *Geobacillus* and *Parageobacillus* species have revealed the presence of several CAZymes suited for biomass valorization [34–38]; these studies have shed light on the molecular mechanisms underlying hemicellulose utilization, underscoring a significant hemicellulolytic potential in their genomes. However, to discover new enzymes of interest for efficient biomass degradation, it is still necessary to identify new strains, characterize their secreted hydrolytic capacities, and analyze their potential on lignocellulosic wastes [34,38,39].

This work reports the whole genome sequencing and assembly of *Geobacillus stearothermophilus* GF16, a thermophilic bacterium previously isolated, and its genome functional annotation to identify key genes involved in lignocellulose degradation [40,41]. Furthermore, it explores the activities of the thermostable GHs secreted by *G. stearothermophilus* GF16 and their efficacy in degrading the hemicellulose component of various agri-food waste biomasses, in comparison to a commercially available enzyme cocktail. The data collected indicate the presence of relevant xylanolytic activities, resulting in the production of xylose as the major hydrolysis product.

Methods

Bacterial growth and genome extraction

A sterile inoculating loop was used to streak *G. stearothermophilus* GF16 on LB agar plates (10 g/L tryptone, 10 g/L sodium chloride and 5 g/L yeast extract, 15 g/L agar) from a frozen glycerol stock. The plates were incubated at 60 °C overnight. Single colonies were inoculated in 10 mL of LB medium and placed at 60 °C on a shaking incubator at 180 rpm for 16 h. Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit (ThermoFisher™) following the protocol recommended by the manufacturer for Gram-positive bacteria. The final yield and quality of the DNA was determined spectrophotometrically using a Qubit Fluorometer (Invitrogen Co.). 200 ng of genomic DNA were then shipped to Novogene Co. (United Kingdom) for microbial whole genome sequencing by preparing a microbial whole genome library (350 bp) for Illumina NovaSeq 6000 and paired-end strategy (PE150 chemistry) generating 1 Gb raw data per sample.

Genome assembly and annotation

A total of 2309,234 reads (average length, 150 bp) was assessed through FastQC v 0.11.9 [42]. Different assemblers, HybridSPAdes v 3.15.3, IDBA-UD v 1.1.3, MEGAHIT v 1.2.9 and Unicycler v 0.4.8 were used, and the results were compared using QUAST v 4.4. The resulting assembly was evaluated for completeness and contamination by means of CheckM v 1.0.18 [43]. The selected assembly (Unicycler) was annotated using Rapid Annotations using Subsystems Technology tool kit (RASTtk) v 1.073 and using the dbCAN3 server (https://bcb.unl.edu/dbCAN2/) for CAZyme annotation. Proksee (https://proksee.ca) was used to draw the genome and the included tool Phigaro for prophage identification [44–46]; a graphical genome view was generated with CGview software [47].

Phylogenetic analysis

To construct the species tree, a set of closely related genomes was imported from the public repository RefSeq available in National Center for Biotechnology Information (NCBI) using Insert Genome Into SpeciesTree-v2.2.0. Due to the vast number of genomes available in NCBI, a subset of public genomes closely related to the user-provided ones was selected. Relatedness was assessed based on alignment similarity to a specific subset of 49 COG domains. Subsequently, the user genome was integrated into our curated multiple sequence alignment (MSA) for each COG family. These curated alignments underwent trimming using GBLOCKS to eliminate poorly aligned sections. Following this, the MSAs were concatenated, and a phylogenetic tree was reconstructed. The average nucleotide identity (ANI) was determined using the Compute ANI with FastANI v0.1.3 and it was reported for the closest genome strains, with a threshold of 80 % [46].

Secretome production in selective growth condition

A frozen (−80 °C) glycerol-stock of *G. stearothermophilus* GF16 was streaked on LB agar medium and incubated for 16 h at 60 °C. A single colony was inoculated in LB liquid medium at 60 °C on a shaking incubator at 180 rpm for 16 h. Cells were collected by centrifugation at 3000 × *g* for 15 min, inoculated at 0.1 OD_{600}/mL in the same amount of: i) rich liquid medium (LB), ii) minimal medium (0.1 % yeast extract) and iii) minimal medium (0.1 % yeast extract) supplemented with 0.1 % xylan (beechwood, Megazyme) in shake flasks, incubated at 60 °C on a shaking incubator at 180 rpm for 4 h for culture grown in LB medium, and for 6 h for the other cultures, corresponding to late exponential growth phase (0.5–0.6 $OD_{600\ nm}$/mL) (Fig. S2) [41]. After growth, the cultures were centrifuged at 3000 × *g* for 15 min, the supernatant was further centrifuged at 17,000 × *g* for 40 min and then

filtered under vacuum through a 0.45 μm nylon membrane (Millipore). The filtrate (i.e. secretome) was concentrated 100-fold using an Amicon ultrafiltration system (Millipore) and a PVDF membrane (cut-off 5 kDa, Millipore) at room temperature and maximum pressure of 75 MPa. After concentration, protease inhibitor cocktail 1X (Roche) was added and the Bradford protein assay was used to estimate the protein content of the secreted proteins, using bovine serum albumin as a calibration standard. Samples were stored at 4 °C for further analysis. Two biological replicates of the secretomes were prepared for each condition.

Detection of GH activities in the secretome of G. stearothermophilus GF16

GH activities in the secretomes of the cells grown under different conditions (described above) were screened against the following substrates: 1 % carboxymethyl cellulose (CMC, Sigma), 1 % arabinogalactan (Megazyme), 1 % glucomannan (Konjac, Megazyme), 1 % beechwood xylan (Megazyme), 1 % pectin from apple (Sigma). Reducing sugars released after the reactions were quantified using the 3,5-dinitrosalicylic acid (DNS) assay performed in 96-well microplates as follows [30,48]: 5 μL of prepared secretome solution were added to 45 μL of a reaction mixture containing the substrates (1 % final concentration) in 50 mM sodium phosphate (Na-P) buffer pH 7.0 and the mixture was incubated at 60 °C for 15 min.

Similarly, to determine optimal pH of the xylanase activity in the 100-times concentrated secretome of *G. stearothermophilus* GF16 grown on minimal medium supplemented with 0.1 % xylan (hereafter referred to as XSEC), the same assay conditions were employed varying only the buffer pH from 3.0 to 9.0 and using xylan as substrate. In detail, sodium citrate buffer (pH 3.0–6.0), Na-P buffer (pH 7.0–8.0), and sodium glycine buffer (pH 9.0) were used. The thermostability of the XSEC xylanase activity was measured at 60 °C and 70 °C at pH 7.0 at different time intervals up to 16 h. Enzyme activity was calculated as U/L, where 1 U is the amount of enzyme(s) capable of releasing 1 μmol of reducing sugars per minute at defined conditions. To calculate the μmol of product(s) released, standard curves of glucose, mannose, xylose were obtained as already described [30]. Each experiment was carried out with two biological and three technical replicates.

Screening of secretome activities on pNP-substrates

The exo-activities of XSEC were determined spectrophotometrically measuring the release of *p*-nitrophenol (*p*NP) using *p*-nitrophenyl-β-D-xylopyranoside (*p*NPβXyl, Biosynth), *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPαAra, Biosynth), *p*-nitrophenyl-β-D-glucopyranoside (*p*NPβGlu, Biosynth), *p*-nitrophenyl-β-D-galactopyranoside (*p*NPβGal, Biosynth) and *p*-nitrophenyl-β-D-mannopyranoside (*p*NPβMan, Biosynth). *p*NP assays were performed in 96-well microplates by incubating 5 μL of secretome with *p*NP-labeled substrates (10 mM) in 50 mM Na-P buffer at pH 7.0, to a final volume of 100 μL for 30 min at 60 °C in a thermomixer (Eppendorf). Reactions were stopped by the addition of 100 μL of 0.5 M Na_2CO_3. The absorbance of released *p*NP was measured at 405 nm in a microplate reader (Synergy H4, Biotek). Secretome activities were expressed in U/L, where 1 U is the amount of enzyme(s) capable of releasing 1 μmol of *p*NP (millimolar extinction coefficient, 18.5 mM^{-1} cm^{-1}) per minute at 60 °C [49,50]. At least three technical and two biological replicates were performed.

Secretome hydrolytic activity on holocellulose-rich biomasses

Agri-food residues: rice husk, roasted coffee beans and wheat bran were ground to uniform size of ~1.2 mm using a blender (Waring® Blenders, Eberbach, Germany) and sieve prior to hydrolysis by XSEC. A typical biomass hydrolysis reaction was performed in a total volume of 375 μL, by resuspending 20 mg of the biomass waste in 350 μL of 50 mM Na-P buffer pH 7.0 and adding 25 μL of XSEC (containing 17 μg protein). The mixtures were incubated at 60 °C under shaking at 200 rpm for 6 and 16 h. Two reactions were set up as controls: a negative control without secretome (only substrate and buffer) and another in which comparable enzymatic units of the secretome were replaced by the commercial enzymatic cocktail Viscozyme® L (Novozymes). The μmol of reducing sugars of all the reaction mixtures were measured by the DNS method [30,48]. The remaining mixtures were boiled at 100 °C in a water bath for enzymatic inactivation, collected at 14,000 × *g* for 5 min, then the supernatants were filtered through 0.22 μm filters and stored at − 20 °C for further analysis.

Quantification of monosaccharides released from hydrolysis with XSEC

To identify the released monosaccharides obtained from the hydrolysis reactions of the agri-food residues with XSEC, high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was employed on an ICS5000 system (Thermo Fisher Scientific). 10 μL of samples were injected on a 4 × 250 mm Dionex Carbopac™ PA1 column with a 4 × 50 mm guard column maintained at 30 °C as previously described [51]. The peaks were then quantified using monosaccharide standards. Final plots were generated using GraphPad Prism 9.

Results and discussion

Genome assembly and functional annotation

To obtain an overall high-quality assembly of the *G. sterarothermophilus* GF16 genome (NCBI genome assembly: ASM2945531v1), a comparison was made between different assemblers. The Unicycler tool gave the best outcome, which consisted of 159 contigs, 5 of them being equal to or greater than 1000,000 base pairs, as determined by QUAST v 4.4 analysis. The total length of the assembly was approximately 3.3 million base pairs, with an N50 value of 57,562 base pairs. The average GC content of the genome was found to be 52.27 %. The completeness of the assembly was assessed with CheckM and was found to be 99.45 %, indicating that most of the expected genomic content was present. The contamination level was found to be 0.55 %, suggesting a minimal presence of foreign DNA. Following the assembly, gene prediction and annotation were performed using the RAST tool [44]. A total of 4387 genes were identified, including 571 predicted non-coding genes which included 5 CRISPR arrays with 115 spacers and 120 repeats. The circularized chromosomal genome was visualized using CGview software (Fig. 1A). A non-coding prophage sequence was also detected in the genome and Phigaro was employed to gain a more detailed annotation of the prophage regions (Fig. 1B) [45].

Phylogenetic analysis of G. stearothermophilus GF16

G. stearothermophilus GF16 was recently isolated and examined for the presence of metal and antibiotic resistance determinants [41]. Ribotyping analyses of 16 S rRNA sequencing and mass spectrometry were conducted to determine the species; however, these analyses were not straightforward to unambiguously identify the species. Therefore, to gain further insights into the taxonomy and confirm the attribution, we performed a genomic phylogenetic analysis based on COG classification. In this analysis, a set of 49 universal genes (COGs) was utilized to compare the genome of *G. stearothermophilus* GF16 with a selected group of 30 closely related genomes obtained from the public KBase database, imported from RefSeq. Multiple sequence alignments for each COG family were appropriately trimmed and concatenated to construct the phylogenetic tree (Fig. 2). The results of this analysis revealed that the isolate GF16 exhibited close relationships with five *Geobacillus spp*. (depicted in light pink in Fig. 2) and was very closely related to the FHS-PPGT111 strain of *G. stearothermophilus* available in the NCBI database (Accession number: NZ_RCTI01000001, Fig. 2). To better assess the relatedness, the ANI was calculated, providing an estimation

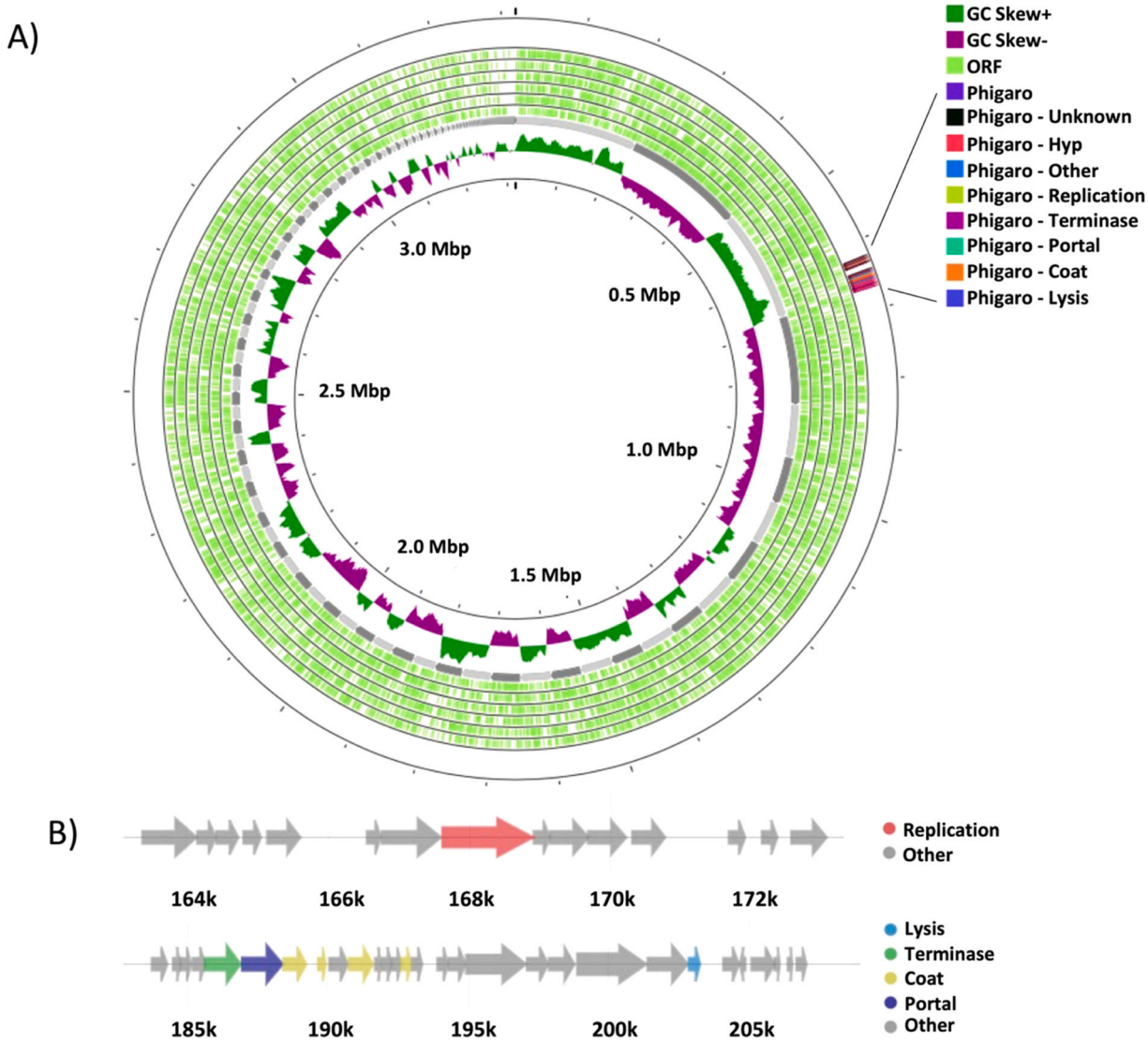

Fig. 1. (A) Circular map of the *G. stearothermophilus* GF16 chromosomal genome performed using the CGview Server. From the center to outside: genome size, with a ring showing the GC skews (positive values in green and negatives in purple); the contigs are represented in grey; the CDSs are represented in light green; and the Phigaro annotations in the outer ring. **(B) Prophage overview of two regions and the linked genes.**

of the mean nucleotide identity between orthologous gene pairs shared by *G. stearothermophilus* GF16 and other microbial genomes in the phylogenetic tree. A cutoff score of >95 % is commonly used to indicate membership within the same species [52]. In this study, *G. stearothermophilus* FHS-PPGT111 was found to have an ANI value of 95.8 % when compared to GF16 (as shown in Table 1) suggesting that the latter is a novel strain different from the one deposited in NCBI.

CAZome of G. stearothermophilus GF16

The search of suitable organisms endowed with saccharolytic activities has led to the discovery of numerous novel isolates, including those found in extreme and isolated environments on Earth [53]. Among these isolates, the genus *Geobacillus* has been reported to possess the capability to degrade complex polymers and to be a valuable source of industrially important enzymes such as GHs, oxidases, and esterases [54]. To assess whether the novel isolate *G. stearothermophilus* GF16 possessed enzymatic activities capable of degrading polysaccharides, the full repertoire of its CAZymes (the CAZome) of its genome was annotated using dbCAN3 [46]. A total of 69 genes were identified as potentially encoding CAZymes and the most abundant classes were GTs (27 genes) and GHs (24 genes).

Additionally, other classes such as CEs, CBMs, and AAs were identified, with 8, 5, and 5 genes respectively (Table S1). To predict the substrate specificity of the identified CAZymes, the tool dbCAN-sub was integrated into the dbCAN3 server. The analysis revealed that all genes containing an identified CBM50 domain were associated with chitin or peptidoglycan degradation, along with a genetic neighbourhood containing a gene encoding a GH18 member (MDF9298404.1). Furthermore, seven genes were found to be linked to starch/sucrose degradation, all belonging to the GH13 family. Regarding lignocellulose deconstruction, two members of AA1 were annotated as multicopper oxidases; interestingly, only one of the genes (MDF9296566.1) exhibited a signal peptide sequence. Moreover, a few enzymes were attributed to cellulose hydrolysis (GH1 and GH4), while a comprehensive set of genes could be associated to xylan degradation. Specifically, two GH10 members coding for endo-1,4-beta-xylanase were identified, one of which appeared to be intracellular, while the other gene (MDF9297509.1) has a predicted signal peptide, indicative of an exported protein. Additionally, putative β-xylosidases were identified within GH39, GH43, and GH52 families which could be capable of generating D-xylose from xylobiose and/or other short-chain

Fig. 2. Phylogenetic tree based on the genome comparison of *G. stearothermophilus* GF16 and related species. The closest strains available in NCBI were used as reference genomes.

Table 1
ANI values from the comparison of *G. stearothermophilus* GF16 with all the reference genomes. No ANI output was reported for a genome pair if the ANI value is below 80 %.

QUERY	REFERENCE	ANI ESTIMATE (%)
G. stearothermophilus GF16	*G. thermodenitrificans* NG80-2	83.7
G. stearothermophilus GF16	*G. icigianus* G1w1	84.8
G. stearothermophilus GF16	*G. subterraneus* E55-1	88.0
G. stearothermophilus GF16	*G. thermoleovorans* RL	91.2
G. stearothermophilus GF16	*G. stearothermophilus* FHS-PPGT111	95.8

xylooligosaccharides. Furthermore, a xylan α-1,2-glucuronidase of the GH67 family was found, that could be involved in the release of glucuronic acid moieties from the terminal non-reducing end of xylooligosaccharides. Additionally, the study identified two putative α-L-arabinofuranosidases from GH43 and GH51. These enzymes, along with four acetyl xylan esterases (Table S1) are known to work in synergy with other xylanolytic enzymes to collectively target complex xylan polymer and/or its hydrolysis products with distinct specificities. This concerted action facilitates the onset of new sites for the enzymatic attack, enabling the subsequent involvement of other enzymes (Fig. 3) [55].

Enzymatic activity profiles of secretomes of G. stearothermophilus GF16

Following the identification of multiple annotated GH enzymes related to xylanolytic activities in *G. stearothermophilus* GF16 genome and its ability to grow in a minimal medium supplemented with xylan as carbon source, we further investigated the ability of this bacterium to produce enzymes related to hemicellulose degradation. Secretomes

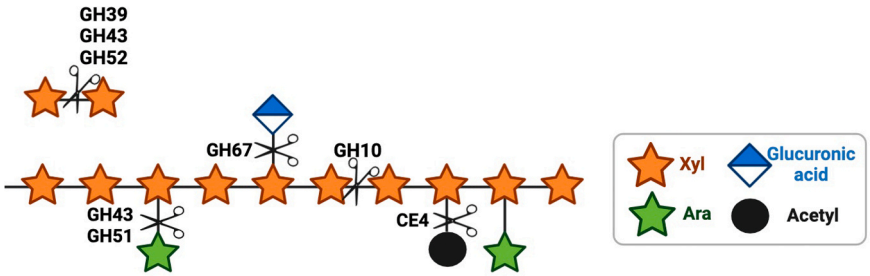

Fig. 3. Representation of decorated xylan backbone with side chains (arabinose, glucuronic acid, acetyl groups). A scheme of enzymatic deconstruction with possible site of hydrolysis is shown together with the putative enzymes: acetyl xylan esterase (CE4), endo-β-1,4-xylanase (GH10), α-L-arabinofuranosidases (GH43 and GH51), α-glucuronidase (GH67), and β-xylosidases (GH39, GH43 and GH52).

obtained from the same amount of cells cultured in LB-rich and minimal yeast 0.1 % media were used to establish a baseline of activity under standard and low nutrient growth conditions, respectively.

The hydrolytic activity profile of the secretome was assessed towards a panel of both native and synthetic polysaccharide substrates, at pH and temperature corresponding to the optimal growth conditions of the microorganism (Fig. S1). As expected, depending on the culture medium in which *G. stearothermophilus* GF16 was grown, the enzymatic activities of the resulting secretome had different properties. Fig. 4A shows that the secretome of cells grown in minimal medium had no detectable GH activity while in XSEC, the 100-times concentrated secretome of *G. stearothermophilus* GF16 grown on minimal medium supplemented with 0.1 % xylan, several different GH activities were identified. Since the activities of the XSEC were always higher than those measured in the secretome of cells grown on LB medium (with the only exception of pectin), we could argue that they were induced by the addition of xylan as a carbon source. Notably, CMCase and arabinogalactanase activities were approximately 3-fold higher for XSEC, whereas glucomannanase and xylanase activities were 5-fold higher. The highest xylanase activity (5×10^3 U/L) was measured in XSEC, suggesting that xylan strongly induces this activity. On the other hand, the uninduced pectinase activity, generally found also in other hemicellulose degrading *Geobacillus spp.*, could play a role in the degradation of L-arabinan polymers associated with pectin [56].

With the aim to better define the GH activities in XSEC, β-xylosidase, α-L-arabinofuranosidase, β-glucosidase, β-galactosidase, and β-mannosidase activities were assessed on different *p*NP-substrates (Fig. 4B); the results showed higher β-xylosidase activity (~ 1.7×10^5 U/L) either in comparison with the other activities in the secretome or with other activities already reported [39,57,58]. The activity profiles for both polysaccharides and *p*NP-labeled substrates align with functional annotation data, which indicate a diverse array of GHs present with predominant xylanase and xylosidase activities. Specifically, CMCase activity could be related to the presence of two putative GH1 enzymes and one from GH4. These families are primarily involved in cellulose degradation, although in the GH1 family other enzymatic activities have been annotated, including β-mannosidase, β-galactosidase, β-glucuronidase, as well as β-xylanase activity [3]. Regarding xylan degradation, the activities can probably be ascribed to the presence of enzymes belonging to GH10, GH39, GH43 and GH52; these putative enzymes, together with the enzyme from GH51, could also be responsible for the high levels of CMC, galactomannan and arabinogalactan hydrolysis observed due to the polyspecific nature of some of these GH families [3]. However, in the functional annotation (Table 2), only three of the putative GHs (GH10, ID number MDF9297509.1; GH13, ID number MDF9296933.1; GH13 ID number MDF9297779.1) had a signal secretion peptide sequence, suggesting that the remaining activities are primarily intracellular. Although it may seem unexpected to have found these activities in the secreted extracellular fraction, such discrepancies are not uncommon; this can be due to imperfect bioinformatic prediction accuracy for the microbial signal peptides which are highly variable, to the presence of secretion systems that lack a canonical signal

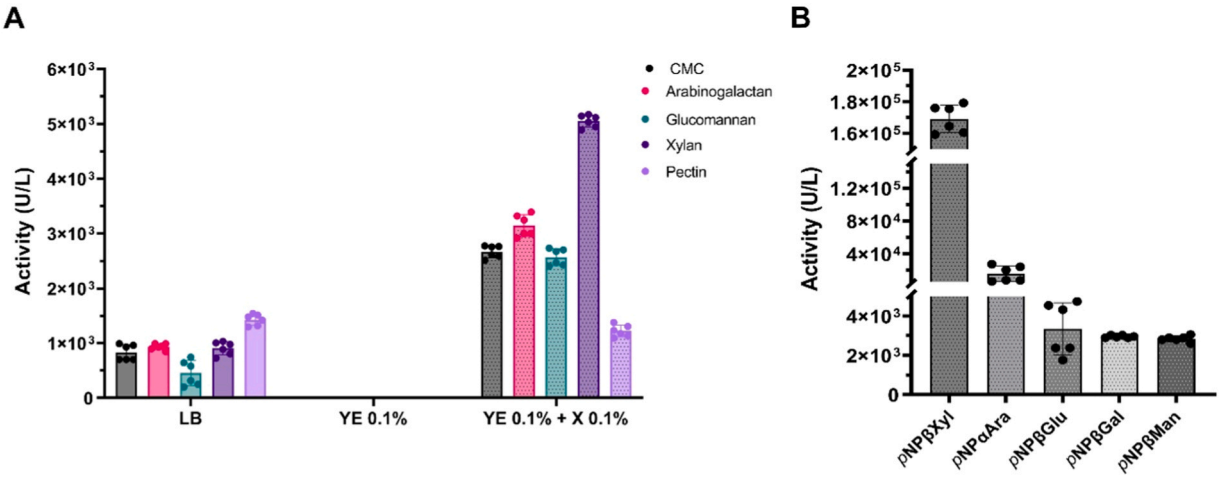

Fig. 4. Secretomes hydrolytic activities profile. (**A**) Enzymatic profiles of CMCase, arabinogalactanase, glucomannanase, xylanase and pectinase activities of the secretomes produced by *G. stearothermophilus* GF16 in response to different media LB medium, Yeast 0.1 % medium and the presence of 0.1 % xylan (X), based on release of reducing sugars detected by DNS method. (**B**) Specific β-xylosidase, α-arabinofuranosidase, β-glucosidase, β-galactosidase, and β-mannosidase activities of XSEC. Individual data points are representative of experiments performed with biological duplicates, each run in at least one technical replicate, with the bars representing the mean and error bars showing the standard deviation.

Table 2
List of GHs identified in *G. stearothermophilus* GF16 using the dbCAN3 server. Biopolymer refers to the putative target polysaccharide, as predicted by dbCAN-sub.

Gene ID	CAZy class	NCBI Annotation	Signal Peptide	Biopolymer
MDF9297482.1	GH1	glycoside hydrolase family 1 protein	N	beta-glucan, beta-galactan
MDF9295640.1	GH1	glycoside hydrolase family 1 protein	N	beta-glucan, beta-galactan
MDF9298170.1	GH4	6-phospho-beta-glucosidase	N	beta-glucan
MDF9297509.1	GH10	endo-1,4-beta-xylanase	Y (1-29)	xylan
MDF9297257.1	GH10	endo-1,4-beta-xylanase	N	xylan
MDF9297690.1	GH13	alpha,alpha-phosphotrehalase	N	sucrose, trehalose
MDF9298498.1	GH13	alpha-glucosidase	N	sucrose, starch
MDF9296929.1	GH13	alpha-glycosidase	N	sucrose, starch
MDF9296933.1	GH13	alpha-amylase family glycosyl hydrolase	Y (1-24)	sucrose, starch
MDF9296226.1	GH13	type I pullulanase	N	sucrose, starch
MDF9296281.1	GH13	1,4-alpha-glucan branching enzyme	N	sucrose, alpha-glucan
MDF9297779.1	GH13	alpha-amylase family glycosyl hydrolase	Y (1-22)	starch
MDF9298404.1	GH18	glycoside hydrolase family 18 protein	N	chitin, peptidoglycan
MDF9297631.1	GH18	glycoside hydrolase family 18 protein	N	-
MDF9295553.1	GH18	LysM peptidoglycan-binding domain-containing protein	N	-
MDF9296458.1	GH23	lytic transglycosylase domain-containing protein	N	peptidoglycan
MDF9297262.1	GH39	xylan 1,4-beta-xylosidase	N	xylan
MDF9297501.1	GH43	β-xylosidase; α-L-arabinofuranosidase	N	xylan
MDF9297245.1	GH51	alpha-L-arabinofuranosidase	N	arabinan
MDF9297256.1	GH52	glycoside hydrolase family 52 protein (β-xylosidase)	N	xylan
MDF9297261.1	GH67	α-glucuronidase; xylan α-1,2-glucuronidase	N	xylan
MDF9298363.1	GH109	Gfo/Idh/MocA family oxidoreductase	N	-
MDF9297845.1	GH130	glycoside hydrolase family 130 protein	N	beta-mannan
MDF9296822.1	GH170	MupG family TIM beta-alpha barrel fold protein	N	-

peptide and also to the intracellular proteins in the secretome, which may result from cell lysis during growth processes [59–61].

The presence of activities not directly linked to xylan degradation in XSEC remains consistent with findings in the literature. Indeed, it is well-documented that members of the *Geobacillus* genus possess genes encoding a variety of enzymes involved in the degradation of diverse carbohydrates; they are often organized into clusters known as polysaccharide utilization loci (PULs) [35,38,62,63]; recent genome analyses of ten *Geobacillus spp*. revealed substantial heterogeneity within this genus, with high variability in the predicted number of gene clusters linked to plant polysaccharide degradation [36]. Thus, the upregulation of gene expression related to xylan metabolism may also lead to the activation of genes encoding enzymes capable of degrading other polysaccharides, as reported also for other organisms such as the fungi *Daldinia decipiens* oita and *Trichoderma reesei* and for the bacterium *Geobacillus* WSUCF1 [38,39,64,65]. Precise identification of the enzymes present in the secretome requires further in-depth proteomic analysis using mass spectrometry approaches. Altogether, these results indicate that *G. stearothermophilus* GF16 is predominantly a xylanolytic organism, and its activities are modulated by the presence of xylan.

Secretome hydrolytic activity on carbohydrate-rich biomasses

To evaluate whether the induced hydrolytic activities could be considered significant for the valorization of agri-food residues and the production of valuable monosaccharides, XSEC was incubated with rice husk, roasted coffee beans and wheat bran, materials with a high and different content of holocellulose [66–68]. As mentioned before, preliminary experiments were conducted using XSEC with the synthetic substrate xylan, to assess optimal pH and define optimal incubation time and temperature that would allow the highest degradation of biomass without losing enzymatic activity (Fig. S1). Afterwards, other preliminary experiments were performed to determine the optimal ratio between protein content of the secretome and solid biomass substrates able to release the highest amount of reducing sugars (data not shown); the conditions resulted to be 0.85 mg of secreted proteins per g of solid biomass. Therefore, after treatment of ground raw biomasses with XSEC, at first the hydrolytic capability was assessed measuring the release of reducing sugars (Fig. 5); in particular, after 16 h of incubation at 60 °C about 1.2 μmol, 0.6 μmol and 0.8 μmol for rice husk, roasted coffee beans, and wheat bran were detected, respectively (Fig. 5B). In order to compare the results of biomass degradation with a commercial enzymatic cocktail, control experiments were carried out by incubating the biomasses under the same experimental conditions, replacing approximately the same total units (~0.153 U) of the XSEC with Viscozyme® L, and evaluating the reducing sugars released. Interestingly, after 16 h, the amount of reducing sugars released from rice husk using XSEC was comparable to that of commercial Viscozyme® L, and for coffee and wheat bran was only about 2-fold lower (Fig. 5B). Thus, the crude XSEC from *G. stearothermophilus* GF16 exhibited comparable performance to commercial Viscozyme® L at 60 °C. These results are in line with those reported for *Geobacillus* WSUCF1 strain, showing hydrolytic activities on both untreated and chemically pretreated corn stover comparable to those of commercial cocktails [39].

Given the significant diversity of the *G. stearothermophilus* GF16 CAZome, we utilized HPAEC-PAD to gain insights into the product profiles of the XSEC to aid in evaluating its potential for saccharification of the aforementioned waste biomasses. The hydrolysis products, in terms of total monosaccharides released from treated agri-food residues are shown in Fig. 6.

In comparison to the hydrolysis performed with Viscozyme® L, treatment with XSEC gave xylose as the predominant product released during the saccharification of biomass residues. Indeed, treatment with the enzymatic cocktail resulted in a highly heterogeneous hydrolysis profile of the biomasses, relatable to the varied stratified organization and percentage of holocellulose of these agri-food residues [67,68]. In this case, saccharification with Viscozyme® L primarily yielded glucose and galactose as the released monosaccharides, while the amount of xylose remained below the detection limit for rice husk and roasted coffee beans (Fig. 6A-B). Notably, treatment with XSEC led to the selective release of xylose as unique monosaccharide in roasted coffee beans, without saccharifying any other compound. In the case of rice husk and wheat bran also glucose was released, albeit at significantly lower concentration compared to the commercial cocktail (Fig. 6B-C). In particular, the xylose released after treatment with XSEC of rice husk and roasted coffee beans is approximately 300-times greater compared to that obtained through Viscozyme® L; regarding wheat bran, the monosaccharide released was about 10-times higher.

The choice of Viscozyme® L, a commercially optimized enzyme cocktail derived from the secretome of *Aspergillus aculeatus*, was deliberate [58]. This choice is based on its documented efficacy in degrading food waste and its minimal environmental impact compared to conventional physical or chemical biomass processing methods [69]. In this study, Viscozyme® L was shown to have remarkable hydrolytic efficacy

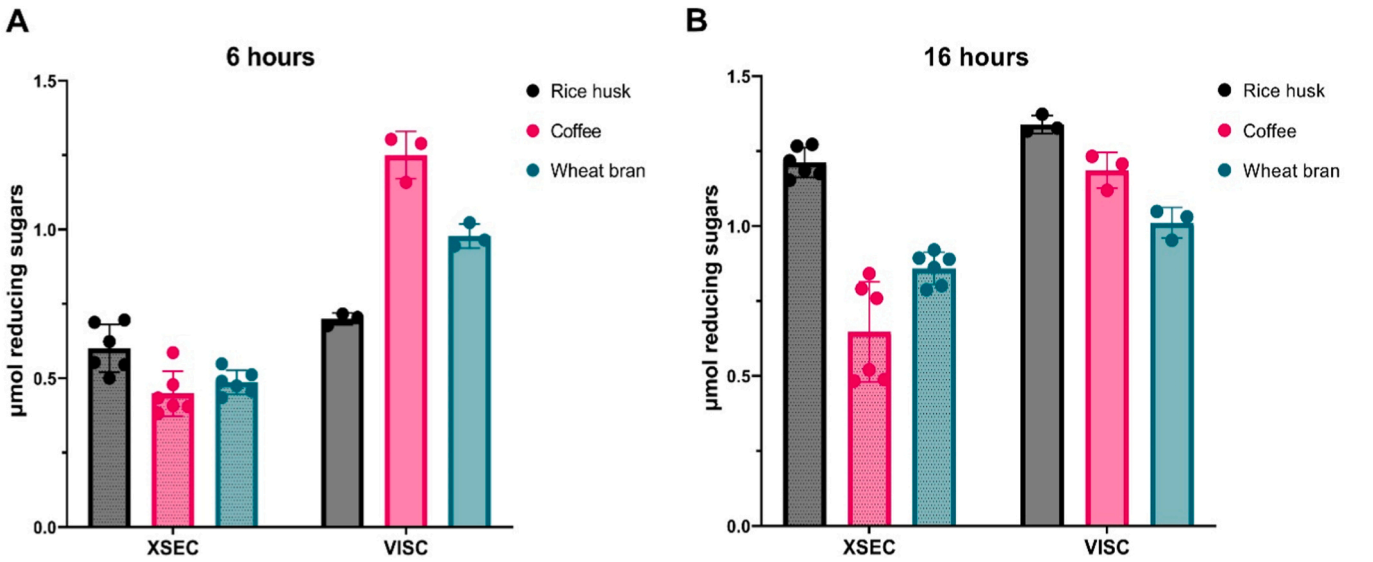

Fig. 5. Hydrolytic activities of XSEC and commercial Viscozyme® L (VISC) on 20 mg of selected biomasses after (A) 6 h and (B) 16 h of incubation at 60 °C and pH 7.0. µmol of produced reducing sugars were quantified by DNS assay as described in the methods. Individual data points are representative of experiments performed with biological duplicates, each run in at least one technical replicate; the bars represent the mean and error bars show the standard deviation.

while maintaining the integrity of the xylan structure for rice husk and roasted coffee beans (Fig. 6A-B). Conversely, XSEC which exhibited a 300-fold increase in xylan hydrolysis activity compared to Viscozyme® L, represents a promising subject for further in-depth analysis and refinement. Thus, this study highlights the potential of thermophilic bacterial secretomes, as previous literature has predominantly focused on the degradative capabilities of fungal secretomes on agricultural and food wastes [18,57,64,65,70]. Furthermore, the utilization of biomass waste from coffee processing provides an additional compelling avenue for exploration.

This work has illuminated the presence of a range of novel enzymes targeting hemicellulose from *G. stearothermophilus* GF16, underscoring its potential use in biotechnological strategies for lignocellulose valorization and composting processes. The results highlight the ability of *G. stearothermophilus* GF16 to produce a highly active and stable xylanolytic cocktail at elevated temperatures when cultured in a commercial carbon source, making it suitable for efficient hemicellulose hydrolysis. The efficacy of this enzymatic set was demonstrated by significant xylose solubilization during the saccharification of rice husk and roasted coffee beans using XSEC, indicating the presence of xylanase and β-xylosidase activity. Its hydrolytic performance on various agri-food waste biomasses is comparable to that reported in the literature for other members of this bacterial genus [34,54,71]. Further characterization of the secretome and enrichment with complementary enzymatic activities, alongside the selection of specific waste biomasses, will enhance the efficiency of the degradation process.

Conclusion

Xylose production is central to several industrial sectors, including biofuel production, biotechnology, and health applications, underscoring its versatility and importance in the bioeconomy. The hemicellulose xylan, characterized by its abundance, cost-effectiveness, and lack of competition with the food sector, holds promise as a sustainable feedstock for biotechnological processes. However, the lack of a comprehensive process or technology for sustainably converting lignocellulosic xylan into value-added products is a significant challenge. Hydrolysis of xylan into usable sugars is a critical step that significantly impacts overall process efficiency. In addition, the presence of hemicellulose in lignocellulosic biomass hinders the accessibility of cellulose to enzymatic hydrolysis, necessitating xylan removal to enhance cellulose bioconversion. Due to the heterogeneous and branched structure of xylan, its enzymatic degradation requires the concerted action of multiple enzymatic activities.

In this study, the genomic and enzymatic analyses of *G. stearothermophilus* GF16 have aimed to fill an important gap in understanding lignocellulose-degrading biocatalytic systems in thermophilic bacteria, offering a promising approach for sustainable xylose production from waste biomasses. Indeed, the treatment of rice husk and especially roasted coffee beans with the xylan-grown *G. stearothermophilus* GF16 secretome, shows the production of xylose as main compound. Utilization of this secretome in conjunction with fermentative strains may provide advantages in optimizing consolidated bioprocesses (CBP) for biofuel production. In addition, rather than focusing on single enzyme production, the generation of a cost-effective secretome with xylose as the major product could be valuable for the synthesis of various xylose-derived compounds, including xylitol, furfural, and xylonic acid. These compounds have applications in industries such as food, pharmaceuticals, and materials science [11,13,72,73]. This can also be significant in processes aimed at degrading the lignocellulosic matrix while preserving the cellulose structure, suggesting the potential use of this treatment for the simultaneous valorization of the cellulose component in these biomasses [10,74].

By identifying lignocellulose-degrading enzymes and demonstrating the inducible GH activities in the secretome of *G. stearothermophilus* GF16, this study lays the foundation for cost-effective enzyme production, biomass conversion, and renewable feedstock valorization. Ultimately, these findings have implications for improving production chains, reducing environmental impact, and advancing the bioeconomy through the utilization of microbial enzymes in sustainable bioprocesses for saccharification of lignocellulosic biomass for fuel and chemical production.

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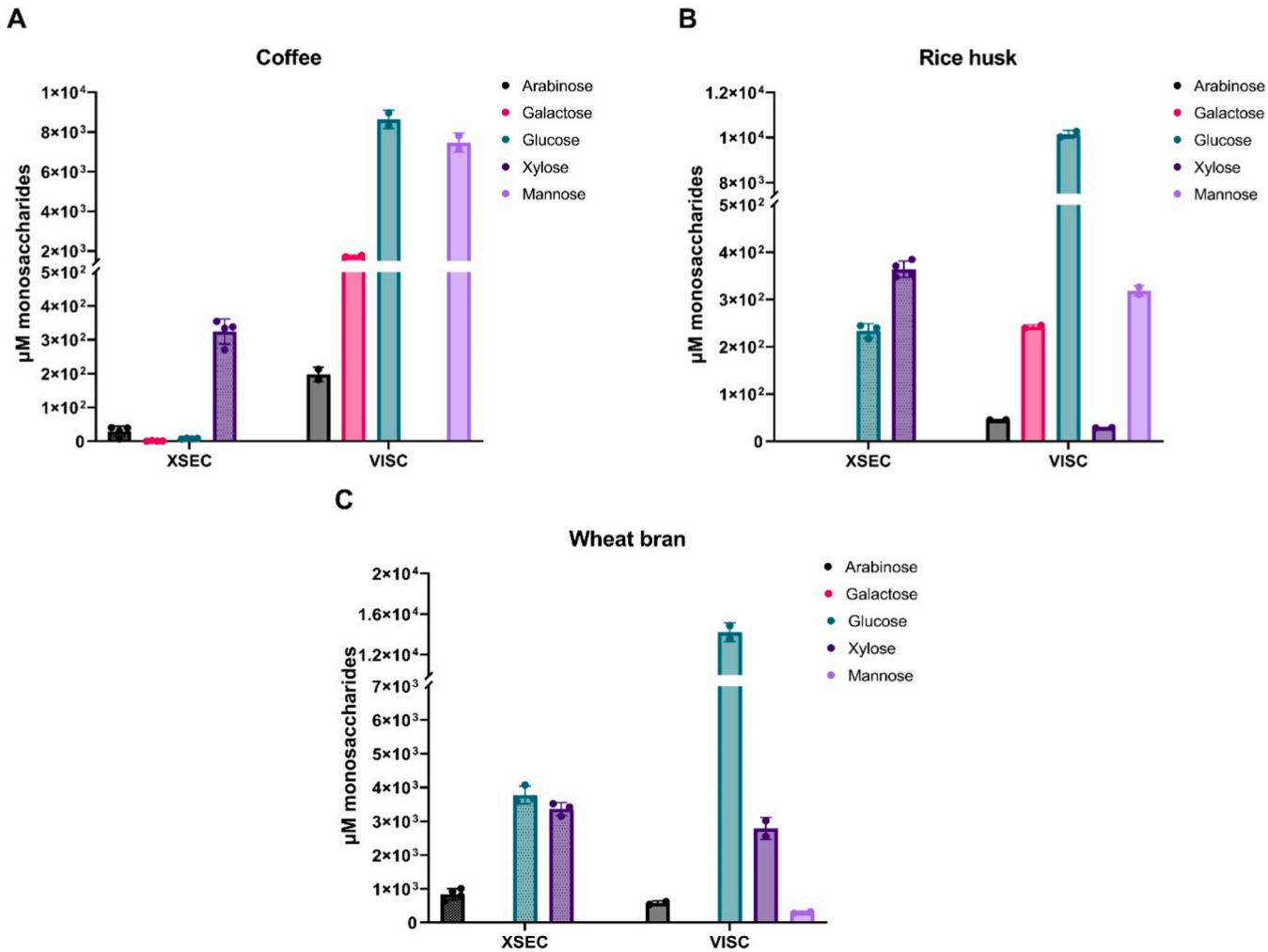

Fig. 6. Profile of monosaccharides released by hydrolysis of agri-food biomasses after 16 h using XSEC and the commercial enzymatic cocktail Viscozyme® L: (A) rice husk, (B) roasted coffee beans and (C) wheat bran. Arabinose, galactose, glucose, xylose, and mannose concentrations were evaluated against monosaccharide standards. As shown in the graphs, it was not possible to detect the presence of certain monosaccharide components for some samples. In those cases, the value for these components was below the detection limit of 10 μM of the standards. Individual data points represent the results of the experiments from biological duplicates (for XSEC, each run in at least one technical replicate). The bars show the mean and the error bars represent the standard deviation among the experiments.

CRediT authorship contribution statement

Patrizia Contursi: Conceptualization. **Danila Limauro:** Conceptualization. **Scott Mazurkewich:** Methodology, Data curation. **Alessia Di Fraia:** Methodology, Data curation. **Johan Larsbrink:** Validation, Conceptualization. **Gabriella Fiorentino:** Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization. **Martina Aulitto:** Writing – original draft, Methodology, Data curation, Conceptualization. **Miriam Carbonaro:** Writing – original draft, Methodology, Data curation, Conceptualization.

Declaration of Competing Interest

All the authors declare no conflict of interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2024.04.002.

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