



A new thermophilic *Parageobacillus* sp. DSM 35475 produces a thermostable hemicellulolytic secretome from spent mushroom substrate and digestate

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ABSTRACT

The development of robust enzyme systems and the use of low-cost feedstocks for lignocellulosic biomass deconstruction remain major challenges for biorefineries. Here, we report a strategy to discover and produce thermostable hemicellulolytic enzymes from industrial microbiomes enriched on spent mushroom substrate (SMS) at 70 °C. A thermophilic bacterium representing a novel species, *Parageobacillus* sp. DSM 35475, was isolated and cultivated on a medium composed exclusively of SMS and digestate, demonstrating the feasibility of producing extracellular enzymes from agro-industrial residues. The resulting cell-free secretome exhibited xylanase activity of 0.22 U/mL, with an optimum at 80 °C and pH 7, while retaining more than 30% of its maximal activity between 60 and 90 °C and more than 50% activity after prolonged incubation at 75–80 °C. More than 40% of the initial activity was preserved after 80 days of storage at 4 °C. HPAEC-PAD analysis of hydrolysis products, together with zymographic and genomic analyses, indicated the presence of a multifunctional hemicellulolytic system comprising endo-xylanase, β -xylosidase, α -L-arabinofuranosidase, and endo-mannanase activities. The secretome hydrolysed beechwood xylan, arabinoxylan and alkali-pretreated SMS, releasing mixtures of mono- and oligosaccharides under thermophilic conditions. Genome annotation predicted a diverse CAZyme repertoire and a dedicated xylan/arabinan utilisation locus consistent with the observed biochemical phenotype. Overall, this study demonstrates the feasibility of producing a thermostable hemicellulolytic secretome from low-cost agro-industrial residues and identifies *Parageobacillus* sp. DSM 35475 as a promising source of thermophilic enzymes for future lignocellulose bioconversion.

1. Introduction

Efficient upcycling of renewable resources will be essential to reduce the reliance on a fossil-based economy and to meet the global climate targets in the coming years [1]. In this context, microorganisms, thanks to their ubiquity and metabolic flexibility, represent suitable tools to increase the sustainability and resource efficiency of biomass-based biorefineries [2]. Among renewable feedstocks, agro-industrial and forestry byproducts represent promising candidates to establish a carbon-neutral bioeconomic model, based on circularity and waste

minimisation principles [3]. These feedstocks are mostly composed of lignocellulose, in which cellulose and hemicellulose fibres are embedded in a matrix of lignin, forming a recalcitrant and hierarchical network, with a reduced number of exposed cleavable bonds [4]. Spent mushroom substrate (SMS), the lignocellulosic by-product from the mushroom industry, is nowadays produced in large quantities. It mainly consists of residual fungal mycelium and disintegrated lignocellulosic biomass (LCB), such as sawdust, corn cob, cottonseed hull, etc., along with amendments (gypsum, peat, lime) and organic material [5]. The improper management of this by-product led to some environmental

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issues such as soil contamination and air and water pollution [6]. For this reason, efforts have been made to develop strategies for its efficient utilisation; these include the use of SMS as an alternative to fertilisers, animal feed or feedstocks to produce bioethanol [7], biogas and volatile fatty acids [8]. Given its high residual content of cellulose (17–41%) and hemicellulose (18–28%) [9], SMS can be also used as substrate to cultivate fungi in solid-state fermentation to produce carbohydrate-active enzymes (CAZymes) [5]. The complex structure of lignocellulose, composed by a fine cellulosic microfibrils, hemicellulose, and lignin, which is the main cause of the LCB's recalcitrance to enzymatic hydrolysis [10]. To overcome this limitation, various pretreatment methods are applied to separate lignin from cellulose and hemicellulose and to allow the enzymes to access the polysaccharides present within the holocellulose fraction [11]. Pretreatment strategies are generally subcategorised into mechanical, chemical, and combined methods. Therefore, the search for thermostable biocatalysts with lower production costs is pivotal to set up a greener lignocellulose processing in biomass-based biorefineries [7].

Thermophilic microorganisms represent an important source of enzymes for industrial applications [12–14] as they can survive in a temperature range between 45 °C and 80 °C, while in extreme cases the hyperthermophiles can live up to 110 °C [7]. These organisms belong to the broader category of extremophiles, which are capable to survive, and even thrive, in extreme conditions, due to peculiar molecular adaptation [15]. Extreme environments include several diverse ecosystems, such as polar environments [76], geothermally heated regions, deep sea [77] and hot springs, as well as some man-made environments such as compost, biogas reactors, or waste contaminated sites [16]. Beyond their importance as sources of industrially-relevant biocatalysts, the study of the genomic characteristics of extremophiles enables us to gain insight into the origin of life, which is expected to have arisen in a high-temperature, anoxic hydrothermal vent [17]. Among all the environmental factors, temperature exerts a strong evolutionary pressure on microorganisms, which shapes the amino acids composition of their proteins and the nucleotide sequences of their genomes [18]. In thermophiles, the adaptation to the high amounts of thermal energy has led to an increase in stability and function of their proteins, to avoid the negative effects of molecular motions [19]. This intrinsic stability, together with their high catalytic efficiency, makes them a greener tool to improve the efficiency and sustainability of biotechnological processes, allowing to operate with low enzyme dosage and, consequently, reducing the overall operational costs. Additionally, thermozymes tend to have a longer half-life and reduce contamination risk compared to mesophilic enzymes [20]. Furthermore, the high-temperature condition at which they can operate also improves substrates solubility and increases diffusion coefficients within bioreactors, enhancing process performance [12]. As a result, the demand for thermostable enzymes continues to grow, especially in second generation biorefineries, where enzymatic hydrolysis is the most effective and sustainable technique for converting complex LCB into fermentable monosaccharides [21].

The complete deconstruction of plant residues requires a synergistic action of several carbohydrate-active enzymes (CAZymes) [22]. Since 1998, thanks to the increasing availability of sequence data, the classification of CAZymes is presented in the continuously updated Carbohydrate-Active enzyme (CAZy) database (www.cazy.org). CAZys are classified into five categories based on the reactions they catalyse [23]. The classification includes glycoside hydrolases (GHs), polysaccharides lyases (PLs), auxiliary activities (AAs), glycosyltransferases (GTs) and carbohydrates esterases (CE). In addition, a pivotal role is played by carbohydrate-binding modules (CBMs), non-catalytic domains that are generally appended to carbohydrate-active enzymes to help them in binding and in the recognition of a notable variety of carbohydrates [24]. Among the CAZymes the main active on LCB are cellulases, xylanases, lignin degrading enzymes, and the peculiar lytic-polysaccharide monooxygenases (LPMOs), that act in synergy to degrade the complex structure of lignocellulose [25]. While lignin-

degrading enzymes are primarily used to modify lignin via oxidative reactions [26] ultimately causing its separation from the hemicellulose (and so exposing also the cellulose), xylanases and cellulases play a central role in the saccharification process via hydrolytic cleavage of the glycosidic bonds. Instead the LPMO help destabilising the surface structure of the microfibrils and cause the chain cleavage by oxidative scission still of the glycosidic bonds [27]. In the last decades, xylanases have gained growing attention thanks to their broad spectrum of application, including textile, animal feed, pulp and paper, pharmaceutical and biofuel sectors [28,29]. Xylanases (O-glycoside hydrolases, EC 3.2.1.x) are produced by a wide range of organisms including bacteria, algae, fungi, protozoa, gastropods and arthropods [30]. Their catalytic activity is directed towards the cleavage of 1,4- β -D-glycosidic linkages in xylan, the major component of hemicellulose. A complete xylanolytic enzyme systems consists of endo-1,4- β -D-xylanases (EC 3.2.1.8), that randomly cleave the xylan backbone, β -D-xylosidases (EC 3.2.1.37), that release xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose, and α -L-arabinofuranosidases (EC 3.2.1.55), α -D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73) and *p*-coumaric acid esterases (EC 3.1.1.-), that serve to remove the side groups of the polymer [31]. According to CAZy database, endo-1,4- β -D-xylanases belong to GH families 3, 5, 8, 9, 10, 11, 30, 43, 44, 51, 98, and 141. Interestingly, some of these GH families, like GH26, 43, 51, 62, and 98, contains bifunctional multi-domain proteins with two catalytic domains, and are called polyspecific families [32]. However, most well-characterized thermostable xylanases are from GH families 10 and 11, with *Bacillus licheniformis*, *Rhodothermus marinus*, *Caldicoprobacter algeriensis*, *Thermococcus zilligii*, *Caldicellulosiruptor kronotskyensis*, *Sulfolobus solfataricus*, and *Pyrodictium abyssi* representing the most studied microorganisms for their production [33]. In particular, *Geobacillus* species have gained increasing interest for bio-based applications not only as cellular factories for heterologous expression of thermostable enzymes but also as source of bioremediators of hydrocarbons, digesters of lignocellulose and as hosts for directed evolution [34]. *Geobacillus* and *Parageobacillus* species are ubiquitous, Gram-positive and rod-shaped cells capable to produce endospores. These bacteria are generally aerobic, facultatively anaerobic and chemo-organotrophic. They can grow in a wide range of temperatures (from 37 to 75 °C), with an optimum at 55–65 °C. Their optimum of pH is around 6.2–7.5, demonstrating a neutrophilic nature [35]. They also exhibit a versatile metabolism as being capable of utilising both C5 and C6 sugars and synthesising different products during fermentation such as acetate, lactate, succinate, and ethanol [36]. In addition to these bacterial and archaeal sources, different thermophilic fungi such as *Thielavia terrestris*, *Rhizomucor pusillus*, and *Corynascus thermophiles* have been exploited to produce thermostable xylanase [37]. It is noteworthy that the adaptation of mesophilic communities to extreme temperatures has led to several advantages linked with industrial lignocellulose degradation. For example, strains of *Bacillus thermoamylovorans*, *Geobacillus thermodenitrificans* and *A. fumigatus* evolved distinctly at the stages of composting showing phenotypes with different thermotolerance and new enzyme expression, exhibiting considerable cellulolytic and hemicellulolytic activities [38]. Moreover, fermentation experiments performed under hyperthermophilic conditions caused the transformation of microbial communities structure and led to the enhancement of extracellular lignocellulolytic activities in dairy manure management, with the genus *Caldicoprobacter* identified as the primary candidate for the increased production of thermozymes [39].

In this study, we isolated a new thermophilic species of the genus *Parageobacillus*, here referred to as *Parageobacillus* sp. DSM 35475, from a microbiome enriched on SMS at 70 °C. The isolate was subsequently evaluated as a platform to produce thermostable carbohydrate-active enzymes by cultivation on agro-industrial residues as the sole components of the induction medium. Its cell-free secretome was then biochemically characterised to assess its hemicellulolytic potential, with particular emphasis on xylan-degrading activities. Temperature and pH

optima, thermostability, and storage stability were determined to define its operational robustness under conditions relevant to biomass conversion. In parallel, electrophoretic and zymographic analyses were used to investigate the molecular distribution of the active enzymes, while HPAEC-PAD profiling of hydrolysis products on model polysaccharides and pretreated SMS allowed us to define the substrate range and infer the coexistence of multiple synergistic hemicellulolytic activities. Finally, genome-based CAZymes annotation provided mechanistic support for the observed phenotype, revealing a specialised repertoire for xylan and arabinan deconstruction, transport, and assimilation. Overall, this work describes a straightforward waste-to-enzyme strategy to exploit industrial microbiomes for the discovery of robust thermophilic biocatalysts and identifies *Parageobacillus* sp. DSM 35475 as a promising candidate for high-temperature lignocellulose deconstruction in sustainable biorefinery settings. The originality of this study lies primarily in the isolation and genome-based characterisation of a distinct *Parageobacillus* strain and in the functional characterisation of the extracellular secretome produced directly from SMS and digestate, rather than in the identification of previously unreported CAZyme families.

2. Materials and methods

2.1. Phylogenetic tree construction

The taxonomic placement of the genome recovered from the microbiome selected on SMS at 70 °C (SRA: PRJNA1047304) was assessed in two complementary steps. First, a phylogenomic species tree was reconstructed using the SpeciesTreeBuilder (v0.1) app implemented in the KBase platform [40,41], run under default settings. Because of the large number of genomes available in NCBI, a subset of public RefSeq genomes closely related to the query was automatically selected based on alignment similarity to a defined set of conserved COG (Clusters of Orthologous Groups) domains. The query genome was then integrated into the curated multiple sequence alignment of each COG family; the alignments were trimmed to remove poorly aligned regions, concatenated, and used to reconstruct the phylogenetic tree, which included a total of 21 reference genomes. Second, to delineate the species boundary, the closest relatives identified in the tree were compared with the query genome using two standardised genome-based metrics: Average Nucleotide Identity (ANI), calculated with the OrthoANu algorithm, and in silico DNA–DNA hybridization (dDDH), estimated with the Genome-to-Genome Distance Calculator (GGDC) version 5. Species boundaries were defined using an ANI threshold of 95–96% and a dDDH cutoff of 70% [44], in line with current genome-based standards for prokaryotic taxonomy [42].

2.2. Media and chemicals

Spent mushroom substrate (SMS) and the digestate from the anaerobic digester were collected from the agro-zootechnical farm “La Torre” (Isola della Scala, Verona, Italy). SMS was oven-dried at 105 °C for about 16 h, ground, and sieved (2 mm, Giuliani Tecnologie, Torino, Italy) to uniform the particle size. The digestate was centrifuged at 6000 ×g for ten minutes to remove solid particles and stored at room temperature. Both SMS and digestate were autoclaved immediately before use to avoid contamination. The SMS and digestate characterisation was previously determined [5].

To revitalise the microbiome previously selected on SMS at 70 °C, its glycerol stock was used to inoculate a seed medium containing 1% w/v of yeast extract (VWR Chemicals, Milano, Italy) and 1% w/v of CMC (VWR Chemicals, Milano, Italy), and incubation at 70 °C overnight.

2.3. Isolation of the new species from the microbiome

The thermophilic microbiome enriched on SMS at 70 °C was

previously subjected to shotgun metagenomic sequencing, from which the metagenome-assembled genome (MAG-70) was reconstructed and identified as a putative novel *Parageobacillus* species based on genome-based taxonomic analyses. To recover the corresponding microorganism in pure culture, the original glycerol stock of the same enriched microbiome was used for colony isolation. Single colonies were isolated from the original microbiome glycerol stock, which was selected at 70 °C. In particular, ten microliters of the glycerol stock were spotted at four positions on a plate containing 2.5 g LB-agar, 1 g NaCl, 1 g CMC, 0.2 g (NH₄)₂SO₄, 0.51 g Na₂HPO₄, 0.33 g NaH₂PO₄, 0.75 mM CaCl₂, 2.5 mM MgCl₂, 1 × trace elements, 1 × FeSO₄, and 1 × vitamin mix. Each spot was then streaked onto a new plate containing the same medium using a sterile inoculating loop and the plate was incubated at 70 °C until visible colonies appeared. Single colonies were picked and used to re-inoculate a new set of plates, which were incubated again at 70 °C until colony growth was observed. These steps were repeated three times to ensure colony purity and reproducibility. A single colony from each plate was subsequently used to inoculate a liquid medium containing 1% yeast extract, 1% CMC, 0.75 mM CaCl₂, 2.5 mM MgCl₂, 1 × trace elements, 1 × FeSO₄, and 1 × vitamin mix. Liquid cultures were grown at 70 °C overnight, and OD₆₀₀ values were measured for each culture. Cultures were centrifuged, and the resulting cell pellets were resuspended in 25% (v/v) glycerol and stored at −80 °C for subsequent analysis.

After confirming comparable colony morphology and growth under the selected cultivation conditions, one representative colony was selected for all subsequent analyses and enzyme production. The strain *Parageobacillus* sp. DSM 35475 has been deposited at DSMZ under the Budapest Treaty. The microorganism and its potential applications to produce thermostable hemicellulolytic enzymes from agro-industrial residues are the subject of a patent application.

2.4. Microbial cultivation for enzyme production

Enzyme production followed the protocol already described in previous works [5,28]. Briefly, a glycerol stock of *Parageobacillus* sp. DSM 35475 was revitalised in the seed medium, by overnight cultivation at 70 °C. Then, the seed culture of *Parageobacillus* sp. DSM 35475 was used to inoculate (in triplicate) at 0.1 OD₆₀₀ mL^{−1} an induction medium prepared by mixing sterile digestate (tenfold diluted in H₂O) and 5% (w/v) of sterile SMS as the sole carbon source and incubated at 70 °C in shake flasks. Cultures were sampled daily and centrifuged at 6000 ×g for 5 min to obtain cell-free secretome (CFS). Enzyme production was monitored by performing colorimetric Azo-xylan assay (Megazyme, Bray, Ireland) to measure the xylanase activity (see Section 2.5). After generally three days of cultivation, the culture that had reached the highest activity was used to inoculate three flasks containing 100 mL of freshly prepared induction medium. Maximum enzymatic activity was generally achieved after two or three days of cultivation. Cultures were centrifuged twice at 6000 ×g for 10 min, to remove the remaining SMS, and filtered through a 0.22 µm nylon membrane by means of a vacuum-filtration apparatus (LLG-Labware, Meckenheim, Germany). The obtained CFS were stored at 4 °C until further use.

To obtain fermentation products, cultures were prepared in duplicate in a medium composed of 1% yeast extract, 1% NaCl, 0.2% (NH₄)₂SO₄, 0.33% Na₂HPO₄ and 0.51% NaH₂PO₄ at pH 7 with the addition of 1% w/v of xylose or glucose. Experiments were performed at 70 °C in baffled Erlenmeyer flasks closed with screw caps and started after the re-inoculation of the seed culture (prepared as in Section 2.2 but without the addition of the polysaccharide). Samples were taken at time 0 and every 24 h, centrifuged at 6000 ×g for 10 min and stored at −20 °C for subsequent analysis.

2.5. Endo-1,4-β-D-xylanase assay

Enzymatic assays were performed according to the manufacturer's instructions (Megazyme, Bray, Ireland), with a few modifications [43].

Briefly, 50 μ L of Azo-Xylan and 50 μ L of *Parageobacillus* sp. DSM 35475 CFSs were preincubated separately for 5 min at 70 °C in an LLG-uniTHERMIX2 pro thermo shaker (Lab Logistics Group GmbH). The reaction was started by mixing the prewarmed Azo compounds with the supernatants in a 1.5 mL Eppendorf tube, followed by incubation at the same temperature for 2 h. To stop the reactions, 250 μ L of precipitation solution (ethanol 95% v/v) were added, each Eppendorf was then vortexed for 6 s and left at room temperature for 10 min. Simultaneously, negative controls were prepared by directly mixing the precipitation solution with the substrates, vortexing and adding the CFSs. Next, to remove the non-hydrolysate polymeric substrate, all samples were centrifuged at maximum speed for 10 min. Finally, 200 μ L of clarified reaction mixture was transferred into a 96-well microplate, and the absorbance was measured at 590 nm using a Synergy Neo2 Hybrid Multi-Mode Reader (Agilent Technologies, Santa Clara, CA, USA). The absorbance of negative controls was then subtracted to each measurement. All the samples were run in triplicate. To determine the enzyme optimal temperature, assays were conducted for 2 h at temperature ranging from 30 to 90 °C. The optimal pH was assessed by adding 10 μ L of various buffer solutions (as reported in [43]) to 50 μ L of Azo-Xylan followed by pre-incubation at the determined optimal temperature. The reaction was then initiated by adding 40 μ L of pre-warmed CFSs to the reaction mixture, followed by incubation. To study the enzyme thermostability, CFSs aliquots were incubated at both their optimal temperature and 5 °C and 10 °C below. At different time points during the incubation, 40 μ L of each aliquot were collected to carry out Azo-Xylan assays at enzymes optimal temperature and pH of activity. Shelf life was evaluated by periodically testing CFSs aliquots previously stored at 4 °C performing Azo-Xylan assays at 70 °C. Data are reported as mean $\pm$ SD of independent replicate.

2.6. Reducing sugars quantification by DNS assay

The concentration of reducing sugars was estimated using the 3,5-dinitrosalicylic (DNS) assay [44,45]. The assay was performed by mixing 35 μ L of *Parageobacillus* sp. DSM 35475 CFSs and 35 μ L of 1% (w/v) xylan solution, resulting in a total volume of 70 μ L. To evaluate the presence of reducing sugars in the CFSs or in the xylan solution, two control reactions were prepared by mixing 35 μ L of CFSs with 35 μ L of dH₂O for the enzyme control, and 35 μ L of 1% (w/v) xylan solution 35 μ L of dH₂O for the substrate control. The samples were incubated at 70 °C for 2 h with constant shaking of 800 rpm. After stopping the reaction by adding DNS solution and incubating the samples at 100 °C for 20 min, the absorbance at 570 nm was measured and used to determine the micromoles of released xylose by means of a calibration curve set up using xylose concentration ranging from 0.5 mg/mL to 4 mg/mL. The absorbance at 570 nm of the two controls was also measured and subtracted during calculation of the released sugar. Data are reported as mean $\pm$ SD of independent replicate. The enzyme activity (U/mL) was calculated by applying the following equation:

$$\frac{U}{mL} = \frac{[\text{released xylose } (\mu\text{mol})]}{[\text{reaction time (min)}] \times [\text{enzyme volume (mL)}]}$$

2.7. Electrophoretic and zymographic analysis

Semi-denaturing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and zymography were performed as reported in [5,46] with few modifications. A non-reducing loading buffer (1.5 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol, bromophenol blue) was added to the samples, and no boiling denaturation was performed before protein electrophoresis separation. The protein marker spanned from 6.5 to 270 kDa (NeoPRO10 Prestained Protein Ladder, Neo Biotech). For zymography, beechwood xylan 1% (w/v) (P-XYLNBE, Megazyme, Bray, Ireland) was added during the preparation of the 12%-acrylamide matrix resolving gel. Following electrophoresis, the gel was washed with a

2.5% (v/v) Triton X-100 solution and incubated at 4 °C in the desired enzyme assay buffer for 10 min. Then, the enzyme reaction was started by increasing the temperature to the enzyme's optimum temperature and incubating for 7 min. The gel was then stained using a 0.1% (w/v) Congo Red solution for 30 min at room temperature. After destaining by washing with 1 M NaCl for 15 min, areas of xylan hydrolysis appeared as clear bands against a darkly stained background.

2.8. Hydrolysis of LCB-derived polysaccharides

The enzymatic activity of *Parageobacillus* sp. DSM 35475 secretome was tested towards Beechwood xylan (P-XYLNBE, Megazyme), wheat flour arabinoxylan (P-WAXYL, Megazyme, ivory nut Mannan (P-MANIV, Megazyme) and spent mushroom substrate (SMS). SMS was subjected to a thermal alkaline pretreatment in 1% NaOH, autoclaved and milled before use. Beechwood xylan, arabinoxylan, carboxymethyl cellulose (CMC), xyloglucan and mannan were mixed with 900 μ L of CMC-70 CFSs and 100 μ L of a 0.5 M Tris-HCl solution pH 7.0 at the final concentration of 1% (w/v). For SMS digestions, CFSs containing microbial enzymes were mixed with 100 μ L of 0.5 M Tris-HCl pH 7.0 and the mix added to the substrate at a final concentration of 10% (w/v). Digestions were performed at the secretome optimal temperature (80 °C) and pH (7.0) under constant shaking of 800 rpm in an LLG-uniTHERMIX2 pro thermos shaker (Lab Logistics Group GmbH). Hydrolysis was stopped after different times by inactivating the secretome at 100 °C for 5 min. After enzyme inactivation, all samples were centrifuged at 10,000 $\times$ g for 5 min, and the hydrolysates were stored at –20 °C for subsequent analysis. Data are reported as mean $\pm$ SD of independent replicate.

2.9. HPAEC-PAD analysis

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was carried out on a Dionex ICS-6000 system equipped with an Ag/Cl reference electrode, and a gold working electrode for the detection [47]. HPAEC-PAD experiments on pure substrates were performed using CarboPac PA210-Fast-4 μ m column. Detection was performed using a gold working electrode and a AgCl reference electrode. The flow rate was set at 0.6 mL/min, and elution of mono- and oligosaccharides was achieved using a potassium hydroxide (KOH) gradient, ranging from 5 to 100 mM. The auto-sampler and column temperatures were maintained at 10 °C and 30 °C, respectively [5,28]. SMS digestions were analysed on a CarboPac PA200–3 mm column, with a flow rate of 0.3 mL/min and the same temperature of the column and auto-sampler. Initial conditions were set to 0.1 M NaOH (eluent A), followed by a linear gradient towards an increasing proportion of a solution of 0.1 M NaOH +0.5 M NaAc (sodium acetate) (eluent B). The gradient reached 60% of solution B in 20 min [28]. All samples were centrifuged, diluted with dH₂O and filtered before use. Standard curves prepared in the range of 25–0.1 mg/L were used to identify and quantify the products in the hydrolysates. All measurements were run in triplicate and reported as mean values $\pm$ SD.

2.10. HPLC analysis

Fermentation products obtained from growth cultures were quantified using the LC-4000 UHPLC (Jasco), equipped with a refractive index detector RI-4030 (Jasco) and a MD-4010 Photometric Diode Array Detector (Jasco). Analytes were separated using a Rezex™ ROA-Organic Acid H + (8%) column (300 $\times$ 7.8 mm; Phenomenex, Castel Maggiore, Italy) coupled with a Carbo-H (4 $\times$ 3.0 mm) guard column (Phenomenex, Castel Maggiore, Italy). The column was maintained at a constant temperature of 80 °C and under an isocratic mobile phase consisting of 5 mM H₂SO₄ (Honeywell, Rodano, Italy) at a flow rate of 0.8 mL/min. All samples were centrifuged, diluted with 5 mM H₂SO₄ and filtered before use. Quantification of glucose, xylose, acetic acid, lactic acid and ethanol was performed with a refractive index and an UV

detector at 210 nm using a standard curve ranging from 0.03 g/L to 6 g/L. All measurements were run in duplicate and reported as mean values $\pm$ SD [48]. Individual data points for each duplicate are shown in Tables S3 and S4.

2.11. Genome annotation and CAZyme prediction

Genome annotation of the MAG70 strain was performed using the RAST server [49]. Functional annotation of carbohydrate-active enzymes (CAZymes) was carried out with the dbCAN3 server (v13), integrating three tools: (i) HMMER, (ii) DIAMOND, and (iii) HMMER against the dbCAN-sub database [50]. Enzymes were classified as CAZymes only when predicted by at least two of these three methods.

2.12. In silico structural and electrostatic analysis of predicted xylan-degrading enzymes

The pH-dependent electrostatic features of the four predicted xylan-degrading enzymes of *Parageobacillus* sp. DSM 35475, PEG2430 (GH43 + CBM91), PEG2438 (GH39), PEG2439 (GH67), and PEG2443 (GH52), were analysed in silico using the previously characterized GH3 β -xylosidase Am β Xyl from *Alicyclobacillus mali* FL18 as a thermo-acidophilic reference [51, 52]. Three-dimensional protein models, predicted with AlphaFold 3 [53], were converted into pH-specific PQR files at pH 5, 6, 7, 9, and 10 using PDB2PQR [54]. Putative catalytic pockets were identified with P2Rank [55], and pocket 1 was selected as the predicted catalytic-site region for the PEG enzymes. Candidate catalytic residues within this region were Glu187/Asp128/Asp15 (PEG2430), Glu160/Glu278/Glu324 (PEG2438), Glu172/Glu299/Asp378/Glu406 (PEG2439), and Glu334/Asp344/Asp494 (PEG2443). For Am β Xyl, the catalytic-site region was defined as all residues within 8 Å of D278 and E516. Electrostatic surface potentials were computed from the PQR files using APBS [56] and visualised in PyMOL 3.1.0 [57], with red and blue indicating negative and positive potentials, respectively. Surfaces were rendered at pH 5, 7, and 9, using identical orientation and graphical settings for each protein. Three charge descriptors were extracted from the pH-specific PQR files and expressed in elementary charge units (e):

total solvent-exposed surface charge (atomic charges summed over residues classified as solvent-exposed by SASA), catalytic-site region charge (residues of P2Rank pocket 1 for the PEG enzymes, or of the 8 Å shell for Am β Xyl), and catalytic-residue charge (candidate residues only).

3. Results

3.1. Taxonomic affiliation and phylogenetic analysis

As reported in our previous work, enrichment cultures were set up to select for mesophilic and thermophilic lignocellulolytic microbiomes, using mushroom spent litter (SMS) as the sole carbon source [5]. Metagenomic analyses of the microbiome selected at 70 °C resulted in the identification of a unique metagenome-assembled genome (MAG), herein referred to as MAG-70. The recovered genome was of high quality, comprising 21 contigs (3.70 Mb total; N50 = 488.4 kb; G + C 43.3%) with 99.45% completeness and 0.61% contamination as estimated by CheckM, qualifying as a high-quality draft according to MIMAG standards [58]. To determine the taxonomic placement of MAG-70, a comprehensive phylogenomic analysis was conducted. We employed a Clusters of Orthologous Groups (COGs)-based approach, which focuses on universally conserved genes. A curated set of closely related genomes was retrieved from the NCBI RefSeq database, and multiple sequence alignments were performed for each COG family. Poorly aligned regions were trimmed, and the resulting sequences were concatenated to construct a robust phylogenetic tree (Fig. 1).

The phylogenetic analysis revealed that MAG-70 is closely related to *Parageobacillus thermantarcticus* M1 (Accession number: [NZ_FOJS01000106]). To assess whether the two strains belong to the same species, we calculated the Average Nucleotide Identity (ANI), a widely accepted metric for genome-level comparison. ANI values exceeding 95% generally indicate species-level similarity [41]. The highest ANI value between MAG-70 and *P. thermantarcticus* M1 was ~91.8%; although below the species-delineation threshold and thus indicative of a distinct species, this value shows that MAG-70 is closely related to *P. thermantarcticus* M1, its nearest phylogenomic neighbour.

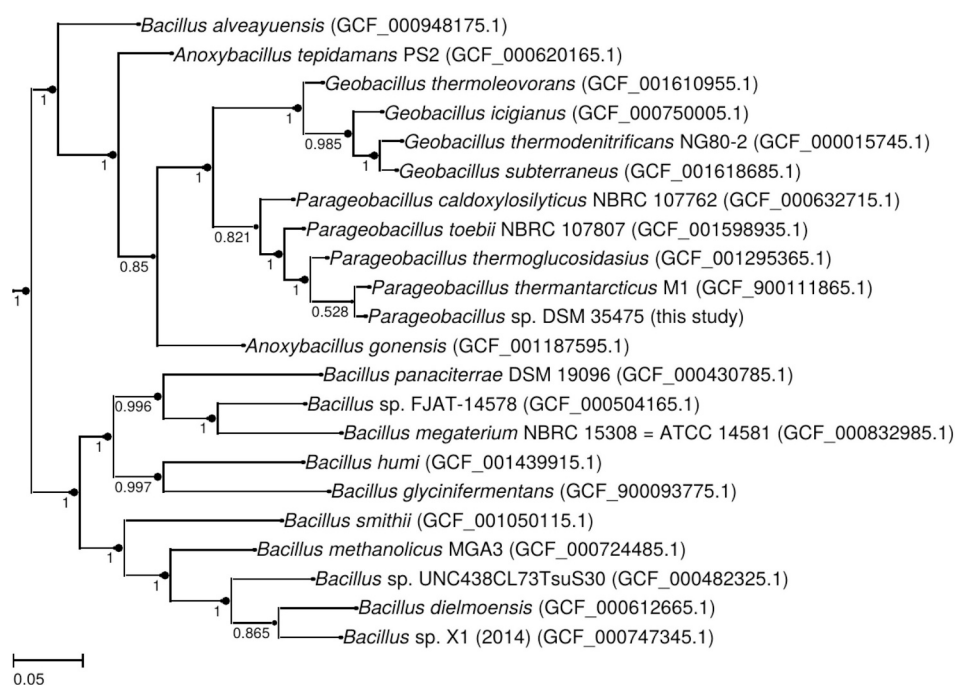


Fig. 1. Phylogenetic tree based on the genome comparison of MAG-70 (i.e., *Parageobacillus* sp. DSM 35475) and other related species of the same Genus. The closest strains available in NCBI were used as reference genomes. Numbers at the nodes indicate SH-like local support values (FastTree 2). The scale bar represents 0.05 substitutions per site.

To support this conclusion, we also performed in silico DNA–DNA hybridisation (DDH) analysis using the Genome-to-Genome Distance Calculator (GGDC). The DDH value obtained via a generalised linear model (GLM) was 11.8%, which is well below the 70% threshold for species delineation. Collectively, these results indicate that MAG-70 represents a novel species within the genus *Parageobacillus*, i.e., *Parageobacillus* sp. DSM 35475.

3.2. Biochemical characterisation of the *Parageobacillus* sp. DSM 35475 secretome

To evaluate thermal stability, the CFS was incubated at 70, 75 and 80 °C, and the residual activity against Azo-xylan was monitored over time (Fig. 2C). At 80 °C, more than 50% activity was retained after 2 h; however, activity declined to 10% after 24 h. At 75 °C, over 50% activity persisted after 5 h, with 20% remaining after 48 h. The highest stability was observed at 70 °C, where the secreted xylanases retained over 70% activity after 5 h and continued to exhibit 30% residual activity even after 96 h. For comparison, Cellic® CTec2 (0.04 FPU/mL in digestate diluted 1:10) was tested at 50 °C (optimum of temperature) and 40 °C. In our experimental conditions (i.e., in the anaerobic digestate), the commercial enzyme cocktail exhibited rapid deactivation, retaining only 20% activity after 1 h at 50 °C, and became completely inactive after 24 h at 40 °C (Fig. 2C). These findings highlight that the *Parageobacillus* sp. DSM 35475 CFS contains thermostable xylanases. It must be pointed out that Cellic® CTec2 was included as a contextual benchmark of operational stability in the digestate matrix. Because the two enzyme preparations were tested at different temperatures and were not normalised based on protein concentration or equivalent xylanase activity, this experiment does not provide a direct comparison of their intrinsic catalytic properties or demonstrate superiority of either preparation.

To assess the storage stability, the CFS was periodically tested by 2 h-long Azo-Xylan hydrolysis assay at the same temperature used to isolate *Parageobacillus* sp. DSM 35475 (i.e., 70 °C), (Fig. 2D). The enzymes retained over 40% of the initial activity for at least 80 days, suggesting long-term stability and highlighting the potential for further

optimisation through purification and formulation.

To ensure strain isolation from the original enrichment culture, single colonies were isolated by streaking into agar plates the glycerol stock of *Parageobacillus* sp. DSM 35475 onto agar plates, as reported in Section 2.3. This procedure led to the production of 4 different glycerol reservoirs, which were subjected to enzyme production trials with the aim of selecting the one with the highest xylanolytic activity. Azo-xylan assay, performed on cell-free secretome (CFS) obtained from the cultivation of *Parageobacillus* sp. DSM 35475, revealed the presence of xylanolytic enzymes, demonstrating the effectiveness of SMS as the only carbon source in the induction medium. To determine the optimal reaction conditions, enzymatic activity was assessed across a temperature range of 30 to 90 °C and in a pH range of 3.0 to 10.0 (Fig. 2A). The *Parageobacillus* sp. DSM 35475 CFS exhibited maximal activity at 80 °C, while remaining above 30% between 60 and 90 °C, confirming the thermophilic nature of enzymes. Optimal pH was identified as pH 7.0 in Tris-HCl buffer. Although the activity optimum was observed at pH 7 (Tris buffer), the secretome retained more than 50% of its maximal activity up to pH 9, demonstrating a moderate tolerance to mildly alkaline conditions rather than true alkalophilicity (Fig. 2B). This broad temperature and pH tolerance suggests that the secretome may be compatible with selected high-temperature or mildly alkaline biomass-processing conditions. However, compatibility with specific pretreatment streams or commercial enzyme formulations was not directly evaluated in this study.

3.3. Xylose-releasing and endo-xylanolytic activity

Xylose was quantified using the 3,5-dinitrosalicylic acid (DNS) assay to measure reducing sugars. Despite this colorimetric assay is widely used to measure reducing sugars, it has some limitations, since it is non-specific for reducing sugars and it can react with all reducing agents. In addition, this assay can lead to an overestimation of sugar concentration, especially in the presence of saccharides with high degree of polymerisation [59,60]. An enzyme unit (U) is defined as the amount of enzyme that releases one micromole (1 µmol) of xylose per minute under

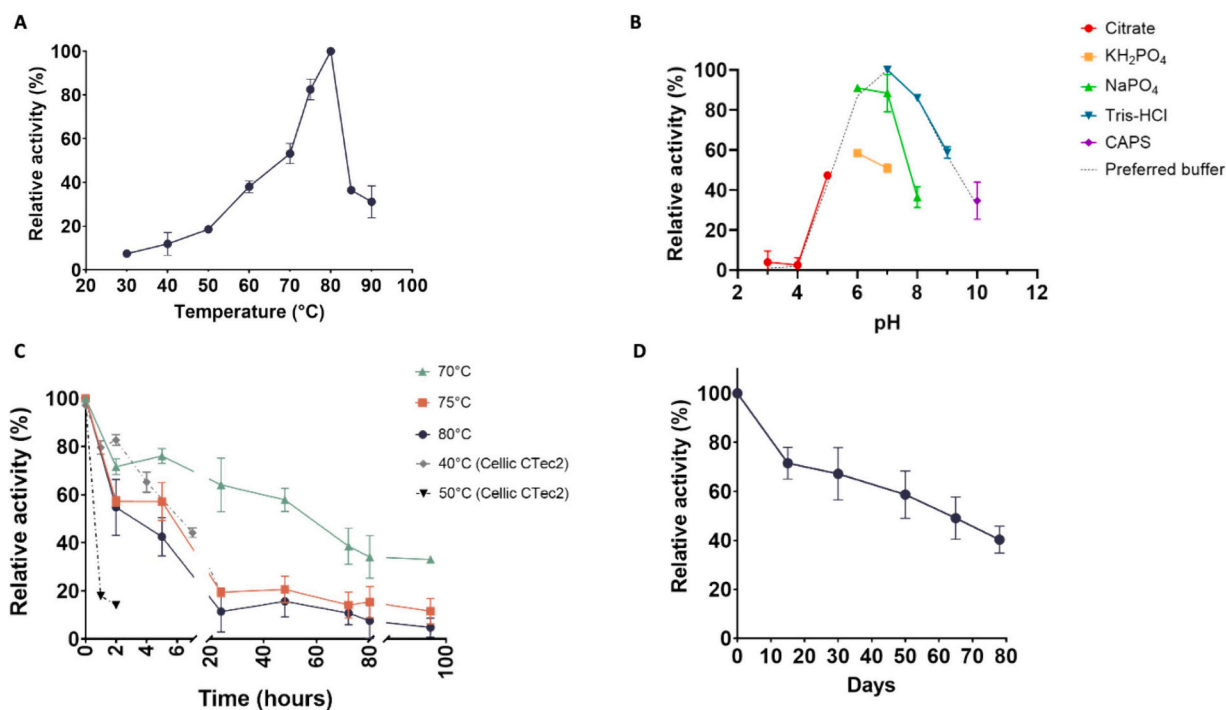


Fig. 2. A) Temperature optimum of *Parageobacillus* sp. DSM 35475 secretome; B) xylanases activity as function of pH; C) thermostability of *Parageobacillus* sp. DSM 35475 CFS and Cellic® Ctec2 when preincubated at different temperatures; D) Shelf life at 4 °C of *Parageobacillus* sp. DSM 35475 secretomes. Reported data are average values of three independent biological replicates using Azo-xylan as substrate [5,28,43].

the assay conditions (80 °C, pH 7.0, 800 rpm, 2 h). Standard samples, with a known concentration of xylose, were used to set up a calibration curve (as in Section 2.6). The average concentration of enzyme units obtained was 0.22 ± 0.01 U/mL. This result is particularly encouraging, given that enzyme production was carried out using an inducing medium composed exclusively of two industrial residues (i.e., SMS and digestate), without any additional nutrient supplementation. Although no optimisation studies were performed, the observed activity demonstrates the feasibility of producing a thermostable hemicellulolytic secretome using agro-industrial residues as the sole components of the cultivation medium. Future process optimization and scale-up studies will be required to assess production yields and industrial applicability.

A key step towards identifying specific enzymes present in the secretomes involves their visualisation by electrophoretic analysis, along with the detection of in-gel activity through zymography. To this end, electrophoretic separations were performed under semi-denaturing conditions. Protein separation and in-gel enzymatic activity detection were carried out via semi-denaturing SDS-PAGE followed by zymography. As shown in Fig. 3A, SDS-PAGE of *Parageobacillus* sp. DSM 35475 CFSs revealed several protein bands between 270 and 66 kDa, and two evident bands, one between 37 and 30 kDa, and another one at 6.5 kDa (Fig. 3A). Zymographic analysis showed that the highest xylanolytic activity was associated with three distinct degradation bands at the topside of the gel. No detectable activity corresponded to the lower molecular weight protein bands (between 37 and 30 kDa, and at 6.5 kDa, Fig. 3B). The molecular weights of the active bands are broadly consistent with several putative xylan-degrading CAZymes. However, since direct proteomic identification of the proteins present in the secretome was not achieved, the correspondence between active bands and specific CAZymes remains tentative and should be interpreted with caution. Neither the number nor the apparent molecular masses of the active bands allow their assignment to specific proteins.

3.4. Hydrolytic potential of the *Parageobacillus* sp. DSM 35475 secretome

High-performance anion exchange (HPAEC) coupled with pulsed amperometric detection (PAD) was employed to analyse the digestion products generated by *Parageobacillus* sp. DSM 35475's CFS, acting on different substrates. In the digestion profile of pure beechwood xylan, peaks corresponding to oligosaccharides with different degree of polymerisation (DPs), from xylobiose (2DP) to xylohexaose (6DP), as well as monomers, can be observed (Figs. 4, S1 and Table S1). During the early stages of hydrolysis, high-degree polymerisation (DP) oligosaccharides were produced in higher amount, reflecting the action of endo 1,4- β -D-xylanases. However, at later time points (T6-T48), the accumulation of these high DP oligosaccharides provided sufficient substrate for exo-

acting enzymes, leading to a rapid increase in lower DP sugars such as xylobiose and xylose monomers (Figs. 4A and S1). This is consistent with the presence of both thermostable endo-1,4- β -D-xylanases and exo- β -1,4-xylosidases (EC 3.2.1.37), that act in synergy to degrade xylan backbone during the time course.

Secretome was also able to digest arabinoxylan, releasing oligosaccharides with different degree of polymerisation, as well as xylose and arabinose monomers, after 24 h of digestion, indicating the presence of α -L-arabinofuranosidases (EC 3.2.1.55) in the *Parageobacillus* sp. DSM 35475 secretome (Figs. 4B, S2). The secretome showed no activity on carboxymethyl cellulose (CMC). In the case of mannan, the chromatogram obtained after 48 h of digestion showed the presence of oligosaccharides (retention time between 10 and 13 min), along with a little quantity of monosaccharides (Fig. S3). This result suggests the presence of endo-mannanase activity within the enzymatic pool, even if it appeared limited under the experimental conditions.

When tested on alkali-pretreated SMS, the *Parageobacillus* sp. DSM 35475 CFSs generated a broad spectrum of peaks, corresponding to xylose and oligosaccharides with different degree of polymerisation (from 2DP to 6DP), as well as higher molecular weight oligosaccharides (Figs. 4C, S4 and Table S1). The ability to degrade structurally recalcitrant substrates under thermophilic conditions highlights the suitability of these secretomes for the critical step of LCB enzymatic hydrolysis in biomass-based biorefineries.

3.5. Fermentation potential of the *Parageobacillus* sp. DSM 35475

To evaluate the anaerobic fermentation ability of *Parageobacillus* sp. DSM 35475, batch cultures were performed in duplicate at 70 °C in yeast extract medium supplemented with xylose or glucose. The content of residual sugars and the production of organic acids were determined by HPLC by sampling the cultures at time 0 and every 24 h. As shown in Fig. 5, *Parageobacillus* sp. DSM 35475 consumed xylose and glucose over time, accompanied by an increase in lactic acid concentration and, with a minor extent, of acetic acid (Tables S2 and S3). The fermentation activity of *Parageobacillus* sp. DSM 35475 was also confirmed by the acidification of the medium during the growth, from pH 7 at time 0 to pH 5–6 at 48 h.

It should be noted that the fermentation experiments were designed to evaluate the metabolic capability of *Parageobacillus* sp. DSM 35475 to assimilate the principal monosaccharides generated by lignocellulose hydrolysis, namely glucose and xylose, rather than to demonstrate a coupled saccharification–fermentation process. A direct evaluation using SMS was beyond the scope of this work, as simultaneous hydrolysis and fermentation under thermophilic conditions would require controlled bioreactor operation with continuous pH regulation. This is particularly relevant because the extracellular hemicellulolytic enzymes displayed maximum activity at approximately pH 7, whereas microbial fermentation progressively acidifies the culture medium, potentially impairing enzyme performance if pH is not actively maintained. Therefore, glucose and xylose were intentionally selected to decouple substrate hydrolysis from sugar utilisation and specifically assess the fermentative potential of the isolate. Combined with the demonstrated production of a thermostable hemicellulolytic secretome capable of depolymerising xylan-containing substrates, these results indicate that *Parageobacillus* sp. DSM 35475 possesses both the enzymatic and metabolic traits required for integrated lignocellulose conversion. However, demonstration of simultaneous hydrolysis and fermentation will require dedicated bioreactor studies and remains an important objective for future work.

3.6. Genomic insights into the hydrolytic potential of *Parageobacillus* sp. DSM 35475

To gain a deeper understanding of the molecular basis underlying the strong xylanolytic and hemicellulolytic activities observed in the

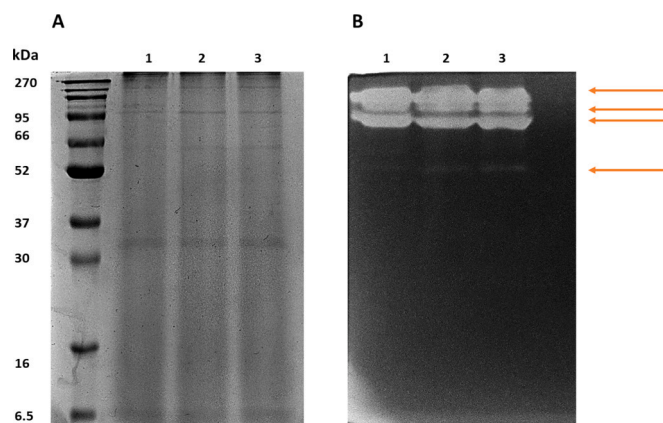


Fig. 3. Electrophoretic analyses of three independent CFSs from *Parageobacillus* sp. DSM 35475, with A) semi-denaturing SDS-PAGE and B) in-gel zymography.

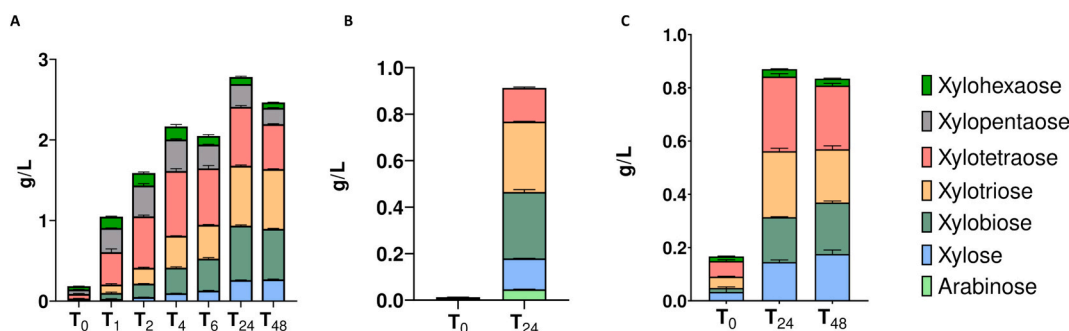


Fig. 4. Analysis of oligosaccharide and monosaccharide released during enzymatic hydrolysis of pure xylan (A), arabinoxylan (B) and alkali-pretreated SMS (C). Error bars represent the standard deviation of three independent experiments.

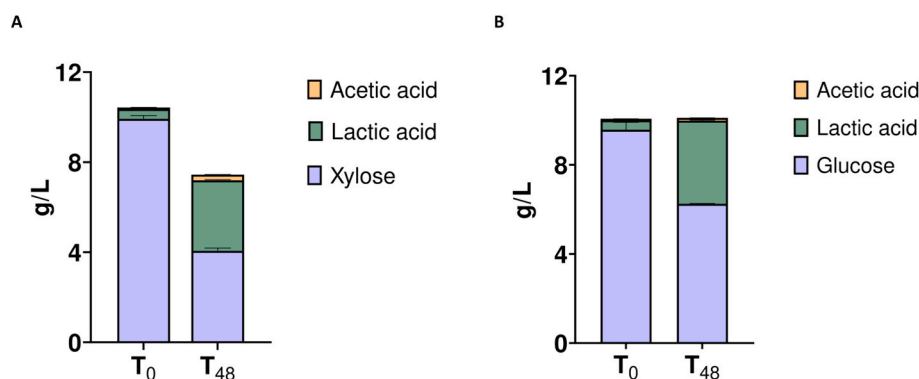


Fig. 5. Analysis of anaerobic fermentation products at 70 °C released by *Parageobacillus* sp. DSM 35475 over time when cultivated in xylose (A) or glucose (B). Error bars represent the standard deviation of two independent experiments (see Tables S2 and S3).

biochemical assays, we extended our investigation to the genomic level to assess whether the enzymatic functions present in the secretome were encoded by specific genetic determinants. This approach allowed us to verify whether the experimentally detected hydrolytic performance reflects a coherent and specialised CAZyme repertoire and to identify genomic modules specifically dedicated to the deconstruction and utilisation of hemicellulosic substrates. Genome annotation revealed a broad and functionally diverse set of 81 carbohydrate active enzymes (Tables S4 and S5), including GHs, CEs, GTs and AAs, many of which are predicted to target major structural components of lignocellulosic biomass such as xylan, arabinoxylan, β -mannan and arabinogalactan. Although the predicted functions are consistent with the observed hydrolysis products and the CAZy family assignments, the catalytic activities of the individual enzymes remain to be experimentally validated. Future heterologous expression and biochemical characterisation will be required to confirm their specific substrate preferences and enzymatic functions. Within this repertoire, a particularly relevant feature is the presence of four xylan degrading enzymes: a putative GH67 α -1,2-glucuronidase (peg.2439) and three putative β -1,4-xylosidases belonging to GH39, GH43 + CBM91 and GH52 (peg.2438, peg.2430, peg.2443). The predicted functions of these proteins are consistent with the progressive accumulation of xylose and low-DP xylo-oligosaccharides. They may contribute, together with as-yet-unidentified secreted *endo*-xylanolytic activities, to the observed hydrolysis profile. Notably, signal-peptide prediction performed within the dbCAN3 annotation indicated that some of these hemicellulose-active GHs lack a predictable canonical signal peptide. While this might suggest release through non-classical secretion routes, this hypothesis was not experimentally tested here; alternatively, the extracellular activities observed may be attributable to other secreted, signal-peptide-bearing enzymes encoded in the genome. This interpretation is supported by the presence of high molecular weight xylanolytic bands were detected in the cell-free

secretome and may correspond to proteins contributing to the initial cleavage of the xylan backbone; however, their molecular identity remains unresolved.

The genome contains several genes predicted to encode activities associated with substituted hemicellulose utilisation, including a GH51 α -L-arabinofuranosidase (peg.2453), a GH53 *endo*- β -1,4-galactanase (peg.2288), and GH2/GH42 β -galactosidases (peg.2284; peg.2289), which are coherent with the release of arabinose and branched oligosaccharides during arabinoxylan degradation and participate in the removal of arabinosyl and galactosyl side chains that limit substrate accessibility. The predicted GH2 β -mannosidase (peg.111) is consistent with a capacity to process mannan-derived oligosaccharides, although it does not identify the protein responsible for the *endo*-mannanase-like activity observed experimentally. Several CE4 deacetylases—including peg.1894, peg.4049 and peg.3748—complement this enzymatic portfolio and likely contribute to the hydrolysis of recalcitrant substrates such as alkali pretreated SMS by removing acetyl groups that hinder enzymatic cleavage.

A particularly striking feature emerging from the RAST annotation (Fig. 6, Table S3) is the presence of a large, contiguous gene cluster spanning peg.2430–2467, which comprises multiple genes annotated as putative xylosidases, such as peg.2430, peg.2438 and peg.2443, an α -glucuronidase encoded by peg.2439, and an extensive series of ABC transporters involved in the uptake of xylosides and xylose, including permeases peg.2440, peg.2441 and peg.2445, substrate binding proteins peg.2442 and peg.2446, and the ATP binding component peg.2444. This region also contains two component regulatory systems responsive to xylan-derived sugars, such as the response regulator peg.2447 and the associated histidine kinase peg.2448. Immediately downstream, a complete putative L-arabinose utilisation operon comprises the α -arabinoside transport components peg.2454–2456, the metabolic enzymes peg.2457 (L-arabinose isomerase), peg.2458 (ribulokinase) and

peg.2459 (ribulose-5-phosphate epimerase), and the additional transport and regulatory components peg.2461–2463 and two putative GntR-family transcriptional regulators (peg.2435 and peg.2460), predicted on the basis of sequence annotation. The co-localisation of these genes within a single genomic region is consistent with the existence of a metabolic module potentially involved in xylan and arabinan deconstruction, uptake and assimilation, although their coordinated regulation and function remain to be experimentally verified.

To place this repertoire in a phylogenetic context, we compared the CAZyme complement of *Parageobacillus* sp. DSM 35475 with that of its closest relative, *P. thermantarcticus* M1, both annotated with the identical dbCAN3 pipeline (Table S5). Overall, DSM 35475 encodes a larger and more diversified set of glycoside hydrolases (36 GH vs 25 GH; 81 vs 71 total CAZyme genes). Most notably, the canonical GH10 *endo*-1,4- β -xylanases present in M1 are absent from the genome of DSM 35475. Rather than relying on these classical *endo*-xylanases, DSM 35475 is predicted to encode a broader array of *exo*-acting and debranching enzymes: in addition to the GH39/GH52/GH67 core shared with M1, it uniquely encodes the arabinan/arabinoxylan-active families GH43 and GH51, a GH53 *endo*- β -1,4-galactanase, and additional GH2/GH3 activities not detected in M1. Consistently, the genes required for L-arabinose utilisation, which are absent in M1, are present and clustered within the utilisation locus of DSM 35475. Taken together, these findings indicate that DSM 35475 may accomplish xylan and arabinoxylan deconstruction through an unconventional, multimodular *exo*-acting and debranching system in the absence of canonical GH10/GH11 *endo*-xylanases — an interpretation coherent with the progressive accumulation of low-degree-of-polymerisation xylo-oligosaccharides

(Fig. 4A) and with the high-molecular-weight active bands detected by zymography. This predicted genomic organisation represents a distinctive feature of *Parageobacillus* sp. DSM 35475 compared with the analysed reference strain, although its functional relevance and coordinated expression remain to be experimentally validated.

Taken together, these genomic features are consistent with a modular hemicellulose-utilisation system and provide a genomic framework for interpreting the experimentally observed phenotype. However, the contribution of the individual predicted CAZymes to the extracellular activities remains to be established experimentally. The strong correspondence between the genomic CAZyme repertoire and the enzymatic activities detected in the secretome consolidates *Parageobacillus* sp. DSM 35475 a robust candidate for high-temperature lignocellulosic biorefinery processes, where thermostability, multifunctionality and efficient processing of hemicellulose derived sugars represent crucial operational advantages.

3.7. Preliminary in silico structural and electrostatic features of the predicted xylan-degrading enzymes

To complement the experimental characterisation of the *Parageobacillus* sp. DSM 35475's CFS, a preliminary in silico structural and electrostatic analysis was conducted on the xylan-active CAZymes PEG2430, PEG2438, PEG2439, and PEG2443 using the thermoacidophilic GH3 β -xylosidase Am β Xyl from *Alicyclobacillus mali* FL18 as a single comparative reference [51, 52]. Because this reference enzyme belongs to a different GH family and represents a single data point, the comparison cannot establish a generalisable electrostatic

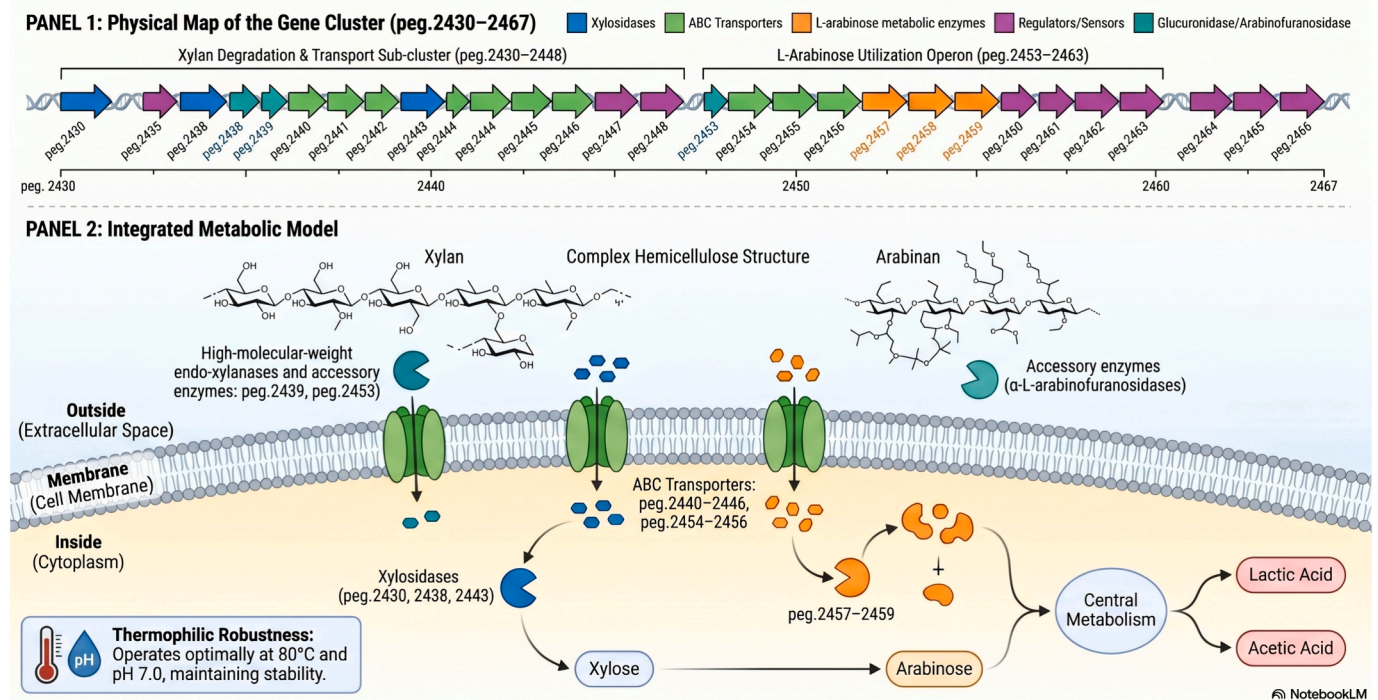


Fig. 6. Genomic architecture and integrated metabolic model for hemicellulose utilisation in *Parageobacillus* sp. DSM 35475. Panel 1: Physical map of the specialised gene cluster (peg.2430–2467). Arrows represent open reading frames (ORFs) and indicate the direction of transcription. Functional modules are color-coded as follows: i) Yellow/Gold: Hemicellulolytic enzymes, including -xylosidases (GH43, peg.2430; GH39, peg.2438; GH52, peg.2443), -glucuronidase (GH67, peg.2439), and -L-arabinofuranosidase (GH51, peg.2453); Blue: ABC transport systems, comprising permeases (peg.2440, 2441, 2445), substrate-binding proteins (peg.2442, 2446), and ATP-binding components (peg.2444) for xyloside/xylose uptake, as well as the -arabinoside transport complex (peg.2454–2456); Green: L-arabinose metabolic operon, encoding L-arabinose isomerase (peg.2457), ribulokinase (peg.2458), and ribulose-5-phosphate epimerase (peg.2459). Red/Purple: Regulatory and sensory systems, including GntR-family transcriptional regulators (peg.2435, 2460), two-component systems (histidine kinase peg.2448 and response regulator peg.2447), and sensory modules (peg.2464–2466). Panel 2: Integrated metabolic model of xylan and arabinan deconstruction. The schematic illustrates the synergistic pathway for hemicellulose valorisation: (1) Extracellular deconstruction of the xylan/arabinan backbone by secreted *endo*-acting enzymes; (2) Import of xylo- and arabinooligosaccharides into the cytoplasm via specialised ABC transporters; (3) Intracellular hydrolysis into monosaccharides by *exo*-acting enzymes; and (4) Metabolic assimilation of xylose and arabinose into the central carbohydrate metabolism [61].

trend and should be regarded strictly as hypothesis-generating. Full APBS-derived electrostatic surfaces at pH 5, 7 and 9, together with quantitative charge profiles, are provided in the Supplementary Information (Figs. S5–S7).

4. Discussion

The development of efficient enzymatic systems for lignocellulosic biomass deconstruction remains a major bottleneck for the large-scale implementation of second-generation biorefineries. In this study, we present a waste-driven strategy that integrates microbial enrichment, strain isolation, and low-cost enzyme production to address both enzyme robustness and production cost. By selecting a thermophilic microbiome on spent mushroom substrate (SMS) at 70 °C, we successfully isolated a novel species of the genus *Parageobacillus*, which we propose here as *Parageobacillus* sp. DSM 35475, and demonstrated its potential as a platform for producing thermostable hemicellulolytic enzymes.

One of the most relevant aspects of this work is the use of agro-industrial residues not only as a source of microbial biodiversity but also as the sole carbon input for enzyme production. The ability of *Parageobacillus* sp. DSM 35475 to grow and secrete active enzymes in a medium composed exclusively of SMS and digestate highlights a promising strategy for improving process sustainability and reducing the dependence on refined substrates. Conventional enzyme production relies on refined substrates and nutrient-rich media, which significantly contribute to overall costs. In contrast, the approach presented here aligns with circular bioeconomy principles, enabling the simultaneous valorisation of waste streams and production of industrially relevant biocatalysts.

The biochemical characterisation of the cell-free secretome revealed a robust and thermophilic enzymatic system, with optimal activity at 80 °C and neutral pH, and a broad operational range spanning 60–90 °C and pH 6–9. These features make this secretome an interesting candidate for future high-temperature biomass processing [11,62]. Notably, the secretome exhibited remarkable thermostability, retaining significant activity over prolonged incubation times at elevated temperatures, as well as long-term storage stability at 4 °C. Under the specific assay conditions used, the *Parageobacillus* sp. DSM 35475 secretome retained detectable activity for longer than the commercial preparation. However, differences in formulation, enzyme composition, loading and assay temperature preclude a direct comparison of intrinsic enzyme performance.

A meaningful comparison can be made with other members of the genus *Parageobacillus*, which has emerged as a relevant source of

thermophilic enzymes for lignocellulose valorisation (Table 1). For instance, *Parageobacillus thermantarcticus* M1 has been reported to produce thermostable extracellular xylanases and β -xylosidases with optimal activities at 70–80 °C, confirming the adaptation of this genus to high-temperature bioprocessing conditions [63]. Genomic analysis of this strain further revealed a broad repertoire of CAZymes involved in hemicellulose degradation and pentose metabolism, supporting its role in lignocellulosic biomass conversion [64].

The hemicellulolytic phenotype observed in *Parageobacillus* sp. DSM 35475 shows several similarities with that previously reported for *Parageobacillus thermantarcticus* M1, one of the best-characterised xylan-degrading species within the genus. Indeed, *P. thermantarcticus* has been reported to produce extracellular xylanase and β -xylosidase activities and has been investigated for the conversion of lignocellulosic feedstocks into xylo-oligosaccharides and fermentable sugars [70]. Consistently, the secretome of *Parageobacillus* sp. DSM 35475 displayed strong xylanolytic activity under thermophilic conditions and contained a set of complementary activities involved in hemicellulose deconstruction, including endo-xylanase, β -xylosidase, α -L-arabinofuranosidase and endo-mannanase activities. However, the present study differs from previous reports in several important aspects. First, *Parageobacillus* sp. DSM 35475 represents a distinct species according to ANI and dDDH analyses. Second, enzyme production was achieved directly from low-cost agro-industrial residues (SMS and digestate), demonstrating a waste-to-enzyme strategy without the need for refined carbon sources. Third, we characterized the extracellular secretome rather than individual enzymes, demonstrating activity not only on model substrates but also on alkali-pretreated spent mushroom substrate. Finally, genome analysis revealed a coordinated repertoire of CAZymes and a dedicated xylan/arabinan utilisation locus linking extracellular polysaccharide deconstruction with intracellular transport and assimilation. Together, these results suggest that xylan degradation is a conserved ecological trait within selected *Parageobacillus* species while highlighting the distinct biotechnological potential of *Parageobacillus* sp. DSM 35475 as a platform to produce thermostable hemicellulolytic enzyme cocktails.

Similarly, *Parageobacillus thermoglucosidarius* has been extensively investigated as a thermophilic microbial chassis for biorefinery applications due to its ability to ferment hexoses and pentoses and its amenability to metabolic engineering [71]. However, previous studies have highlighted that extracellular proteolytic activity may negatively affect enzyme secretion in this species, representing a limitation for direct enzyme production [72]. In contrast, the strong extracellular xylanolytic activity observed for *Parageobacillus* sp. DSM 35475, together with the clear detection of active high-molecular-weight proteins in the secretome, is consistent with the hypothesis that this strain is

Table 1
Comparison of the biochemical properties of CAZyme repertoire across *Parageobacillus* species.

CAZyme	<i>Bacillus</i> sp.	Temperature optimum (°C)	pH optimum	Thermostability	References
1,4- β -D-xylan xylanohydrolase	<i>B. thermantarcticus</i>	80	5.6	Stable at 60 °C for 24 h Half life at 70 °C after 24 h Half life at 80 °C after 50 min	[63]
1,4- β -D-xylan xylohydrolase	<i>B. thermantarcticus</i>	70	6.0	Stable at 60 °C for 1 h 2.5% initial activity at 80 °C after 1 h	[63]
SPS-0 xylanase	<i>B. sp. strain SPS-0</i>	75	6.0	Stable at 60 °C Stable at 70 °C for 4 h (with xylan) Loss of activity at 80 °C in 15 min	[65]
Endo-xylanase	<i>B. sp. strain DUSELR13</i>	75	7.0	>50% of the activity at temperatures 50, 60, and 70 °C for 48, 38, and 13 days respectively	[66]
Alkali-stable xylanase	<i>G. thermodenitrificans</i> A333	70	7.5	88% of activity at 60 °C and up to 97% in the pH range 7.5–10.0 after 24 h	[67]
Endo-xylanase	<i>G. sp. WSUCF1</i>	70	6.5	50 and 60 °C, retaining 82% and 50% of its original activity, respectively, after 60 h	[68]
Alkali-stable xylanase	<i>G. thermoleovorans</i>	80	8.5	3 h at 60 and 70 °C with $T_{1/2}$ of 60 and 10 min at 80 and 90 °C, respectively	[69]
Cell free secretome	<i>Parageobacillus</i> sp. DSM 35475	80	7.0	>50% activity for hours in the range 70–80 °C	This work

well suited for secretome production under thermophilic conditions. Although zymographic analyses, substrate hydrolysis profiles, and genome annotation consistently support the presence of a multifunctional hemicellulolytic system, definitive assignment of the observed activities to individual proteins was not possible. Several attempts were made to identify the active components of the secretome by LC-MS/MS, involving different research groups with expertise in proteomic characterisation. However, because the secretome was produced in a complex medium composed exclusively of spent mushroom substrate and digestate, substantial co-extraction of residual organic material and other interfering compounds prevented reliable protein identification. Therefore, the association between the active zymographic bands and the predicted CAZymes should be regarded as putative.

Nevertheless, the convergence of biochemical assays, HPAEC-PAD hydrolysis profiles, zymography, and genome-based CAZyme annotation provides complementary evidence consistent with the presence of the predicted hemicellulolytic activities. Interestingly, although most characterized bacterial GH10 and GH11 xylanases have molecular masses between 20 and 50 kDa, the active zymographic bands were detected at apparent molecular weights above 95 kDa. Because zymography was performed under semi-denaturing conditions, the apparent molecular masses may reflect multidomain proteins, stable oligomeric assemblies, incompletely dissociated complexes, or anomalous electrophoretic migration. These possibilities cannot be distinguished without direct protein identification and should therefore be considered alternative hypotheses rather than confirmed mechanisms. Given the bacterial origin of the strain, extensive glycosylation is considered less likely to explain the observed migration pattern. However, these interpretations remain tentative until the active proteins can be conclusively identified by future proteomic analyses.

Comparative genomics across the genus further supports these observations. Recent pan-genome analyses have shown that *Parageobacillus* species harbour diverse and highly variable hemicellulose utilisation loci, reflecting adaptation to different ecological niches and substrate availability [73]. In this context, the gene cluster identified in *Parageobacillus* sp. DSM 35475, comprising multiple xylosidases, an α -glucuronidase, arabinose-utilisation genes, and associated transport and regulatory systems, points to a highly integrated metabolic module for hemicellulose deconstruction and assimilation. The overall agreement between genomic predictions and biochemical observations reinforces the functional specialisation of this strain and suggests an efficient coordination between extracellular hydrolysis and intracellular metabolism.

Complementary in silico structural and electrostatic analyses of the four predicted xylan-degrading enzymes provided an additional, hypothesis-generating layer to the genomic picture. The predicted surface electrostatics showed a progressive shift towards a more negative global potential as pH increased for all four PEG enzymes. However, compared to the thermo-acidophilic control enzyme Am β Xyl, the *Parageobacillus* enzymes underwent a comparatively more limited increase in overall negative surface charge under neutral-to-alkaline conditions. This more moderate electrostatic variation may contribute to maintaining favourable interactions with polysaccharidic substrates at pH values above 7, a pattern that is broadly consistent with, though not direct evidence for, residual catalytic activity in alkaline environments; given the single-reference-enzyme design, this observation remains exploratory.

Beyond stability, the enzymatic profile of the *Parageobacillus* sp. DSM 35475 secretome is consistent with the presence of a multifunctional hemicellulolytic system. Chromatographic analyses demonstrated the progressive conversion of xylan into a mixture of oligosaccharides and monomers, indicating the synergistic action of *endo*- and *exo*-acting enzymes. The release of arabinose during arabinoxylan hydrolysis further supports the presence of accessory enzymes targeting side-chain decorations, while the partial hydrolysis of mannan suggests an extended substrate range. Importantly, the secretome was also active on

alkali-pretreated SMS, confirming its ability to act on structurally complex and industrially relevant lignocellulosic substrates. This broad substrate specificity is a desirable feature for enzyme cocktails intended for real biomass processing [74,75].

Another interesting feature emerging from this study is the fermentative capabilities of *Parageobacillus* sp. DSM 35475. Our results indicate that *Parageobacillus* sp. DSM 35475 appears to possess both the enzymatic machinery required for hemicellulose hydrolysis and the metabolic capability to assimilate its principal hydrolysis products. Whether these complementary traits can be successfully integrated into a single consolidated bioprocess remains to be demonstrated in dedicated bioreactor studies under controlled operating conditions and therefore represents an important direction for future research.

From a process perspective, the most distinctive feature of *Parageobacillus* sp. DSM 35475 is its ability to produce active, stable enzyme cocktails directly from low-cost agro-industrial residues, without the need for refined substrates. While previous studies on *Parageobacillus* species have primarily focused on purified enzymes or engineered strains, the present work demonstrates, at laboratory scale, the feasibility of producing an active thermostable secretome using SMS and digestate without refined carbon substrates. This proof-of-concept suggests a potential residue-based production route, but scalability, productivity, environmental benefits and economic viability remain to be quantitatively established. A notable aspect of this study is that enzyme production was achieved using SMS and digestate as the sole components of the cultivation medium. However, the present work was designed as a proof-of-concept study aimed at demonstrating the feasibility of producing a thermostable hemicellulolytic secretome from agro-industrial residues. No process optimization, bioreactor-scale cultivation, productivity assessment, mass balance, or techno-economic analysis was performed. Therefore, although the use of waste-derived feedstocks may offer opportunities for reducing raw material costs, further studies will be required to quantitatively evaluate process performance and economic viability.

While previous studies on *Parageobacillus* species have primarily focused on purified enzymes or engineered strains, the present work demonstrates the feasibility of producing a thermostable hemicellulolytic secretome directly from low-cost agro-industrial residues under laboratory conditions. Future studies should focus on improving enzyme production yields, identifying and characterising the individual secreted enzymes, validating secretome production under controlled bioreactor conditions, and assessing the operational compatibility of the enzyme cocktail with industrial lignocellulosic biorefinery processes. These aspects will be essential to fully evaluate the translational potential of *Parageobacillus* sp. DSM 35475.

5. Conclusions

This study demonstrates the feasibility of a waste-driven approach for the discovery and production of thermostable enzymes using thermophilic microorganisms adapted to lignocellulosic environments. Through enrichment and isolation from an SMS-based microbiome, we identified a novel species, *Parageobacillus* sp. DSM 35475, capable of producing a robust and multifunctional hemicellulolytic secretome using low-cost agro-industrial residues as the sole carbon source.

The resulting enzyme cocktail exhibited optimal activity at 80 °C and neutral pH, with broad operational stability and long-term shelf life. Biochemical and chromatographic analyses revealed a synergistic system capable of degrading both model substrates and pretreated lignocellulosic biomass, while genomic insights confirmed the presence of a specialised CAZyme repertoire supporting these activities.

Importantly, this work highlights how industrial microbiomes can be harnessed not only as a source of biodiversity, but also as a functional reservoir of enzymes already adapted to harsh and process-relevant conditions. The combination of thermophilic robustness, substrate versatility and laboratory-scale production from low-cost feedstocks

identifies *Parageobacillus* sp. DSM 35475 as a candidate for further evaluation in high-temperature biomass-conversion processes.

Future studies will focus on the identification, heterologous expression, and optimization of key enzymes within the secretome, as well as on the development of tailored enzyme formulations for specific biomass feedstocks. In addition, further integration of hydrolytic and fermentative capabilities may enable the design of more efficient and consolidated bioprocesses for lignocellulose valorisation.

CRedit authorship contribution statement

Luca Bombardi: Writing – original draft, Investigation, Data curation. **Valerio Sabellico:** Investigation. **Luca Zuliani:** Investigation. **Giovanni Gallo:** Writing – review & editing, Investigation. **David Cannella:** Writing – review & editing, Supervision. **Martina Aulitto:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Data curation. **Salvatore Fusco:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that a patent application related to the microorganism and its applications described in this study has been filed. The authors have no other financial or personal conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2026.153852>.

Data availability

Sequencing data have been submitted to SRA database, project PRJNA1047304.

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