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Comparative secretome dynamics of *Beauveria bassiana* under solid-state and submerged fermentation

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ABSTRACT

Beauveria bassiana (Bb) is a fungal entomopathogen that is popular in the industry as a biopesticide. Recently, some Bb strains have been demonstrated to have the ability to produce lignocellulose-degrading enzymes. Hence, to explore this fungus beyond its biocontrol applications - especially in bioprocesses which may involve its lignocellulolytic machinery- its differential secretome response to lignocellulosic biomass degradation under submerged fermentation (SF) and solid-state fermentation (SSF) conditions was evaluated for the first time using a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) based approach. Initially secreted Bb proteins under the two conditions were harvested, and subjected to LC-MS/MS. Subsequently, the differential expression of the proteins was analysed while their carbohydrate-active enzymes (CAZymes), functional and gene ontology were annotated. Results revealed that the Bb secretome contained 613 (40 upregulated) and 1162 (301 upregulated) proteins during SF and SSF cultivations, respectively. Approximately 70 % of the proteins were identified with the general secretory (Sec) pathway, while showing low probabilities of being transmembrane proteins. The secretomes were principally composed of carbohydrate-active enzymes (CAZymes), proteases, metabolic proteins and structural proteins. Gene ontology also highlighted biomass deconstruction, facilitated by the plethora of Bb CAZymes as well as its proteases, as a prominent metabolic activity in the secretomes. Biological processes, such as microtubule cytoskeleton organisation, the glucan and cellulose catabolic process, as well as the carbohydrate metabolic process, were observed to be more pronounced in SSF relative to SF. The 147 and 236 CAZymes annotated during SF and SSF cultivation were observed to act on a wide range of polysaccharides, including cellulose, starch, xylan, lignin, chitin, and peptidoglycans. Hence, these findings demonstrate the higher complexity of Bb lignocellulose degradation under SSF relative to SF, thus further highlighting Bb as a model entomopathogen with potential in biomass pretreatment for biofuel production and the production of important enzymes for biomass valorisation and other sustainable bioprocesses.

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Introduction

As one of nature's most prominent recyclers of organic material, filamentous fungi (FF) play a significant role in several geochemical processes and nutrient cycling. These groups of organisms, which are remarkably diverse in terms of their morphology, physiology, and ecology, are known to easily metabolise a wide range of substrates, especially lignocellulosic biomasses and organic residues [1]. Studies at the molecular level have since established that the genomes of FF contain more protein-encoding genes when compared to other fungal groups, a phenomenon attributed to their morphological complexity and wide array of secondary metabolites [2]. Due to these merits, FF have long been exploited as cellular factories in the production of antibiotics, bioactive compounds, pigments, and enzymes, among other applications [1]. The renewed quest for a more sustainable environment has maintained FF at the forefront of bioprocessing, as their hydrolytic enzymes have demonstrated immense applicability and feasibility, especially in the biofuel industry, as well as in the food, feed, detergent, textile, and pulp and paper industries [3]. Hence, FF-facilitated enzymatic transformations can be considered key factors in the pursuit of achieving some of the UN Sustainable Development Goals (SDGs), such as Affordable & Clean Energy (SDG 7), Responsible Consumption and Production (SDG 12), as well as Industry, Innovation, and Infrastructure (SDG 9). More specifically, bioprocesses facilitated by FF possess the capacity to advance the UN SDG targets of substantially increasing the share of renewable energy in the global energy market (Target 7.2); enhancing sustainable industrial processes (Target 9.4); sustainable management and efficient use of natural resources (Target 12.2); and reducing waste through recycling and reuse (Target 12.5).

Generally, the *Aspergillus, Fusarium, Penicillium, Rhizopus*, and *Trichoderma* genera are regarded as the most popular FF utilised across different industries, especially in the production of organic acids, enzymes, protein substitutes, and antibiotics [4]. More recently, another FF, *Beauveria bassiana* (Bb), has gained attention because of its various biotechnological potentials. The entomopathogenic fungal endophyte -which has long been used in the industry as a biocontrol agent– was noted to be a remarkable source of a wide array of important bioactive compounds as well as a versatile producer of industrially important enzymes [5]. For instance, recent investigations have demonstrated that a strain of Bb (*B. bassiana* SAN01) produces a wide array of glycosyl hydrolases, including β-glucosidase, cellulases, xylanase, amylase, and polygalacturonase, at significant levels [6]. Similar observations have also been made with other strains of B. bassiana; for example, *B. bassiana* IBCB 66 was recorded to produce endocellulase, exocellulase, chitinase, and β−1,3-glucanase in copious quantities under solid-state fermentation conditions [5]. Xiao, Ying [7] had earlier provided remarkable insights into the genome of Bb, showing that its 33.7-megabyte genome contained 145 genes for carbohydrate-active enzymes (CAZymes). Subsequently, the CAZyme secreting potential of Bb strains was further demonstrated by elucidating the transcriptome under different trophic conditions [6]

Although genomic sequencing provides the overall genetic blueprint, while transcriptomic data measures gene expression, it has been noted that neither genomic nor transcriptomic data can explicitly elucidate the levels of active, functioning proteins in the cell [8]. In essence, the proteomic profiling of an organism provides a more comprehensive picture of its active and functional proteins, with a focus on the practical output and efficiency of bioprocesses [8]. According to Du and van Wezel [9], the unambiguous understanding of an organism's "protein secretion mechanism" is pertinent for its optimal application as a "cellular factory" for the production of important proteins and metabolites. To this end, proteomic analyses of various fungal secretomes (externally secreted proteins) have been used as an effective approach for bioprospecting industrial enzymes, as seen in recent cases of *Aspergillus fumigatus* [10] and *Trichoderma erinaceum* [11]. Typically, the secretomes of FF are considered industrially significant as their extracellular protein production translates into easier product recovery. More often than not, the physiology of FF is adapted to grow on moist, solid, water-insoluble substrates, hence, their cultivation under solid state fermentation conditions is believed to be closer to their natural conditions [12]. Consequently, the need for direct contact and the stress imposed by the solid-state environment directly influence the composition and complexity of their secretome [13]. There are some indications that proteins secreted under solid-state fermentation (SSF) conditions differ in abundance and, in some cases, are differentially localised compared to those secreted under submerged fermentation (SF) [13,14]. For example, *Aspergillus oryzae*, was shown to display a broader secretome with unique extracellular protein profiles and altered cell wall dynamics under SSF relative to SF [14]. Hence, having identified Bb as a notable industrial enzyme source, its industrial potential - especially in the valorisation of lignocellulosic biomass and other important bioresources- could be further enhanced by the enumeration of the full complement of its secreted proteins using a proteomic approach.

To this end, this work aimed to enhance the applicability of Bb by elucidating its secretome while metabolising lignocellulosic biomass. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been identified as an efficient tool for elucidating the proteome of industrial microbes, based on its high sensitivity, specificity, comprehensiveness, and versatility. It provides significant insights into the physiology, metabolism, and behaviour of an organism under different conditions [15]. The LC-MS/MS-based proteomic approach has contributed significantly to the improvement of microbial strains for various biotechnological applications including the identification of pathways to enhance enzyme production and optimisation of culture conditions for industrial applications. Consequently, advanced LC-MS/MS-based secretomics was employed to provide a detailed, comparative, and industrially relevant functional map of *B. bassiana* SAN01's lignocellulose degradation under SSF and SF, which are considered the two most popular fermentation approaches in bioprocessing. This approach provides the mechanistic evidence necessary to extend the current understanding of Bb beyond its established role in insect pathogenesis, underscoring its underexplored potential in biomass deconstruction and sustainable enzyme biotechnology. Furthermore, it offers new perspectives into how fungal secretion patterns are influenced by the fermentation mode, suggesting that environmental context is a major determinant of enzyme induction and extracellular protein complexity. The demonstrated potential of *B. bassiana* SAN01 to produce a wide range of lignocellulose-degrading enzymes under SSF as well as SF, using agro-residues, accentuates a locally adaptable approach for converting agricultural waste into value-added products. Thus, in addition to being aligned with the UN SDGs, this work provides a sustainable bioprocessing platform

that supports strategic objectives of sustainable industrialisation and green growth as outlined in the African Union's Agenda 2063. Based on the available literature, this study is the first to report the differences in the secretome of any entomopathogenic fungi- Bb inclusive- under both SSF and SF of lignocellulosic biomass as well as to detail the CAZymes profile of Bb secretome.

Methodology

Beauveria bassiana growth conditions

Beauveria bassiana SAN01 (Gene Accession Number: MN544934), previously isolated in its endophytic phenotype from onion leaves, was obtained from the culture collection of the Department of Biotechnology and Food Science at the Durban University of Technology, South Africa. The fungus was grown on potato dextrose agar (PDA) slants at 30 °C for 5 days and the inoculum was prepared by suspending the spores in sterile 0.1 % Tween 20 solution, achieving a final concentration of 1×10^7 spores/mL.

Solid-state and submerged fermentation

Two different fermentation conditions, SSF and SF, were evaluated to compare the proteomic expression of *B. bassiana* SAN01. Wheat bran was chosen as the substrate, being one of the most utilised lignocellulose biomass for bioprocessing. For SSF, 4 g of wheat bran was used, and the moisture content was maintained at 80 % using a mineral salt solution. In the SF, 4 g of wheat bran was suspended in 100 mL of optimised mineral salt solution at pH 5.0. Subsequently, both media were autoclaved for 20 min at 121 °C and inoculated with 1 mL spore suspension containing 1×10^7 spores/mL. The media were then incubated at 30 ± 2 °C for 10 days; however, SF was carried out under constant agitation at 120 rpm. Following the 10-day incubation period, samples were subjected to protein extraction.

Extraction of extracellular proteins

Subsequent to SSF, 30 mL of 0.1 M sodium acetate buffer (pH 5.0) was added to the flask and agitated at 120 rpm and 30 ± 2 °C for 1 h. Subsequently, the mixture was filtered through muslin cloth and the filtrate was centrifuged at 12 000 × *g* for 20 min at 4 °C. The supernatant thus obtained was used as the enzyme in subsequent analysis. For SF, the media was centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatant was similarly collected for subsequent analysis. The extracellular proteins from solid-state and submerged fermentation conditions were quantified using the Lowry method [16], normalised, and subjected to proteomic analysis.

LC-MS/MS analysis of the fungal secretome

On-bead protein digestion

The protein samples were diluted in 100 mM Tris–HCl containing 100 mM NaCl and 1 % SDS, followed by reduction with 5 mM Tris (2-carboxyethyl)phosphine (TCEP) in 100 mM Tris buffer for 1 h at 60 °C. Cysteine residues were carbamidomethylated with 20 mM iodoacetamide in the dark for 30 min in 100 mM Tris/SDS at room temperature. Subsequently, the reaction was quenched with 20 mm dithiothreitol (DTT), and the samples were diluted two-fold with binding buffer (200 mM ammonium acetate, 30 % acetonitrile, pH 4.5). The protein solution was added to MagResyn HILIC magnetic particles (Resyn Biosciences) prepared according to the manufacturer's instructions and incubated for 30 min at room temperature. After binding, the supernatant was removed, and the magnetic particles were washed twice with 95 % acetonitrile (ACN). Subsequently, the magnetic particles were suspended in 25 mM ammonium bicarbonate containing trypsin at a final enzyme-to-protein ratio of 1:50. Digestion was carried out for 18 h at 37 °C, the peptides were then extracted, and the magnetic beads were washed once with 50 μL of 1 % trifluoroacetic acid. Finally, the samples were dried and re-suspended in 2 % ACN and 0.1 % formic acid (FA) in water at 1 mg/mL, for LC-MS analysis.

Liquid chromatography

Liquid chromatography was performed using a Thermo Scientific Ultimate 3000 RSLC system (Thermo Scientific, USA), equipped with a C18 trap column (5 mm x 300 mm, Thermo Scientific, USA) and an analytical C18 column (CSH 25 cm × 75 mm, 1.7 μm particle size (Waters, USA). The solvent system consisted of solvent A (2 % ACN in water with 0.1 % FA) and solvent B (100 % ACN with 0.1 % FA). The samples were loaded onto the trap column using solvent A at a flow rate of 2 mL/min from a temperature-controlled autosampler set at 7 °C. Loading was performed for 5 min before the sample was eluted onto the analytical column. The flow rate was set to 300 nL/min, and the gradient generated was 5.0 % −30 % solvent B for 60 min, followed by 30–50 % solvent B for 60–80 min. Chromatography was performed at 45 °C and the outflow was delivered to the mass spectrometer.

Mass spectrometry

Mass spectrometry was performed using a Thermo Scientific Fusion mass spectrometer (USA) with a Nanospray Flex ionisation source. Samples were introduced through a stainless-steel nano-bore emitter. Data acquisition was performed in positive mode, with spray voltage set to 2.1 kV and ion transfer capillary set to 295 °C. Spectra were internally calibrated using polysiloxane ions at *m/z* 445.12003. MS1 scans were performed using the Orbitrap detector set at 60,000 resolution over the scan range of 375–1500 m/*z* with the automatic gain control (AGC) target set to the standard value. MS2 acquisitions were performed with monoisotopic precursor selection for ions with charges ranging from +2 to +7, with error tolerance set to ±10 ppm. Precursor ions were excluded from

fragmentation for a period of 60 s. Precursor ions were selected for fragmentation in the higher-energy collisional dissociation (HCD) mode, using quadrupole mass analysers with an HCD energy setting of 30 %. Fragment ions were detected in the Orbitrap mass analysers, set to a resolution of 30 000. The AGC target was set to standard and the maximum injection time to 100 ms. All data was acquired in centroid mode.

Analysis of differentially expressed proteins

The mzML data files were subjected to database search, de novo sequencing, and quantification using PEAKS Studio 11 software (Bioinformatics Solutions Inc, Canada). The search was performed against the UniProt ID Map of *B. bassiana* (www.uniprot.org). The standard parameters considered were: Instrument Triple TOF, acquisition—DDA, fragmentation method—CID, error tolerance of up to 10 ppm for precursor, fragment ion tolerance 0.2, enzymatic specificity of trypsin digestion, reduction, and alkylation of proteins (fixed modification—carbamidomethylation; variable modification—acetylation N-term, oxidation M), maximum allowed variable post-translation modification per peptide 3 [17]. Extracted ion chromatograms (XIC) from up to 2 most intense unique peptides were averaged to infer protein abundance. Relative protein quantification was performed and statistically tested using the PEAKS Q module (ANOVA method). Only proteins exhibiting a fold change of $\geq$ 1.0 were considered differentially abundant between the SSF and SF samples [18].

Signal peptide and transmembrane topology

SignalP5.0 (using cut-off default) was utilised for the prediction of secreted proteins in the Bb secretomes (https://services.healthtech.dtu.dk/services/SignalP-6.0/) while the Transmembrane Helices, Hidden Markov Model (TMHMM) tool (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) was used to elucidate the membrane topology.

CAZyme annotation of B. bassiana secretome

Carbohydrate active enzymes (CAZymes) in the SF and SSF secretomes were identified and classified by the dbCAN3 meta server. This web-based meta server (http://bcb.unl.edu/dbCAN2/index.php) annotates CAZymes using three integrated search tools, viz., HMMER (http://hmmer.org/), DIAMOND (https://github.com/bbuchnk/diamond) and HotPep [19]. Only those proteins successfully annotated by at least two of the integrated tools were curated as CAZymes.

Functional annotation of B. bassiana secretome

Proteins identified in the secretomes were subjected to gene ontology annotation and functional enrichment analysis using the Functional Enrichment Analysis tool (FunRich, version 3.1.4). Furthermore, the differentially secreted proteins were analysed using the Database for Annotation, Visualisation and Integrated Discovery (DAVID, https://david.ncifcrf.gov/home.jsp).

Statistical analysis

Three biological replicates were prepared for each fermentation condition and each biological sample was analysed in three technical replicates to account for instrument variability and enhance reproducibility. The one-way analysis of variance test was used for all data and means (for three independent experiments) and compared according to the Tukey–Kramer test at 5 % level of significance.

Results

Overview of B. bassiana proteome

In this study, the LC-MS/MS analysis of *B. bassiana* SAN01 secretome subsequent to lignocellulosic biomass utilisation revealed the true expression profile of its proteins, especially the repertoire of its biomass-degrading enzymes. The average number of proteins identified during cultivation on agricultural residue was found to be 613 and 1162 under SF and SSF conditions, respectively. While the number of common proteins between the two conditions was 538, the total number of proteins was 1237 (see Supplementary Figure S1). The secreted Bb proteins were observed to have molecular weights ranging from 4.14 to 211.89 kDa, with amino acid lengths ranging from 40 (elongation factor 1-alpha) to 5132 (HC-toxin synthetase) residues. The extracellular proteins produced by Bb under both SF and SSF were classified based on their abundance in the major protein classes, viz., carbohydrases, proteases, structural proteins, metabolic proteins and other classes (see Supplementary Figure S2). In this study, the "other" class consisted of minor protein groups, including defence proteins, transport proteins, transcriptional factors, ribosomal proteins, as well as many unclassified proteins. Besides the "other" group, the highest number of proteins in both SF and SSF was observed in the carbohydrase group, followed by the metabolic proteins, proteases and structural proteins, in descending order. It was also observed that the SSF secretome had a significantly higher amount and diversity of proteins across all classes, except in the protease class, where both conditions expressed a similar number of proteins.

Correlation between SF and SSF

The correlation data generated by PEAKS Studio indicate a significant correlation between the samples within each fermentation condition/group. It was observed that the correlation within the SSF group was the strongest, with values ranging from 0.98 to 0.99. In contrast, the correlation within the SF group was relatively lower, with values ranging from 0.82 to 0.92 (see Supplementary Table S1). Expectedly, a low inter-condition correlation was observed between biological replicates of the SF and SSF group, ranging from ~0.27 (SF 2 vs SSF 1 and SSF 3) to ~0.62 (SF 1 vs SSF 2) (see Supplementary Table S1). In summary, a correlation of ~0.44 was observed between the SF and SSF groups, indicating a significant difference in the extracellular protein profile of Bb under the two fermentation conditions (see Supplementary Figure S3).

Signal peptide and transmembrane proteins in Bb secretome

Approximately 70 % of the Bb proteins were predicted to contain the signal peptides with probabilities 0.9–1.0, further establishing their extracellular localisation and transportation via the classical Sec pathway. A typical example of such is the glucan 1,3-beta-glucosidase protein (A0A0A2VZ64). The absence of a signal peptide in the remaining proteins, including alpha-L-rhamnosidase (A0A0A2VHX5), suggests that they may exhibit significant intracellular functions.

Results show that >90 % of the secretome have zero predicted transmembrane helices, indicating that they are less likely to be transmembrane proteins but are strictly extracellular. It was observed that most of the Bb proteins with transmembrane helices were involved in the transport mechanism of the fungus. These include the major facilitator superfamily (MFS) profile domain-containing protein, choline transporter-like protein, putrescine import ATP binding protein, chemotaxis protein and calcium-transporting ATPase 1 with 12, 9, 7, 5 and 5 transmembrane helices, respectively.

Comparative secretome analysis

The differentially expressed proteins in the secretome of Bb SAN01 were evaluated by comparing the protein expression profiles while degrading wheat bran under SF and SSF conditions. The principal component analysis (PCA) showed that the SF and SFF secretomes were clearly separated (Fig. 1a). Notably, the PCA plot was able to elucidate approximately 80 % of the total variance within the dataset; specifically, the principal components 1 and 2 accounted for 61 % and 20.2 %, respectively (Fig. 1a). Thus, the biological replicates of each fermentation condition fall into non-overlapping clusters, indicating significant differences between the groups. It was also observed that only 341 out of the total 1237 expressed proteins were differentially expressed ($p < 0.05$). Furthermore, the SSF secretome, which contained 301 upregulated proteins, was observed to have a significantly higher number of upregulated proteins compared to the SF, with log2 fold changes ranging from 1.6 to 64. On the other hand, 40 proteins were identified as being upregulated in SF, with log2 fold changes ranging from 2.3 to 11. Consequently, approximately 25 % of the total proteins in the SSF secretome were upregulated compared to the 6.5 % of the SF secretome. The higher number and proportion of extracellular proteins expressed in SSF relative to the SF conditions is also illustrated in the volcano plot, which computed the overall distribution of proteins between the two experimental groups (Fig. 1b).

Similarly, the dynamic modulation of protein expression in Bb SAN01 under the two fermentation conditions was elucidated in the global heatmaps, which were constructed at $p \leq 0.01$ and $p \leq 0.05$ (Fig. 2). The similarities of the biological replicates within the same fermentation group were established via the protein expression clustering. Furthermore, the heatmaps also highlighted the significant differences between SF and SSF via hierarchical clustering, revealing that the differentially expressed proteins could be classified into

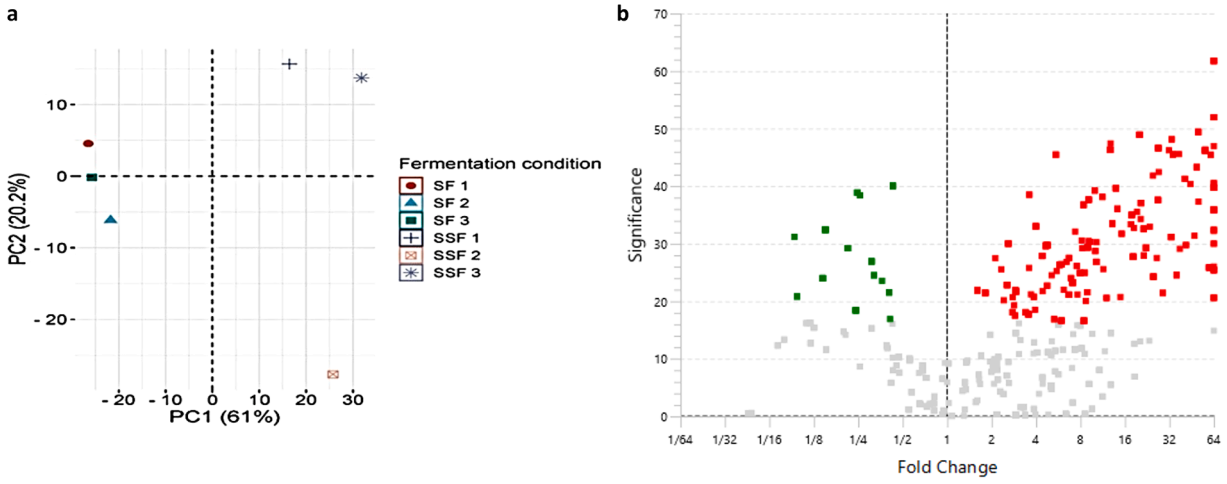

Fig. 1. Near here.

two major clusters. While one of these clusters was observed to comprise the SSF upregulated proteins such as A0A2S7Y9S7 (pectate lyase superfamily protein domain-containing protein), A0A0A2VWW0 (cell wall mannoprotein), A0A2S7YJ86 (lipocalin/cytosolic fatty-acid binding domain-containing protein), J4WF58 (cell wall protein), as well as A0A2S7Y8C9, which is an uncharacterised protein. The other main cluster group consists of all the proteins that were recorded as being upregulated in SF, e.g., A0A0A2VDY4 (carboxypeptidase C), J5K3A3 (C6 transcription factor), and J5JQF0 (feruloyl esterase).

Upregulation of B. bassiana SAN01 proteins under solid-state fermentation (SSF) conditions

As stated earlier, 301 out of the total 1162 proteins were recorded to be upregulated in the SSF group relative to the SF group (see Supplementary Table S2). In this study, the SSF upregulated proteins belonged to diverse groups, including carbohydrases, defence proteins, oxidases, proteases, phosphatases, topoisomerases, structural proteins, and nucleases, among others (see Supplementary Table S2). Generally, proteins directly involved in carbohydrate hydrolysis and specifically lignocellulosic biomass deconstruction were found to be upregulated under SSF conditions. In addition, a wide range of proteolytic enzymes was also found to be upregulated. A summary of some of the selected upregulated SSF proteins shows the presence of carbohydrases, including pectate lyase, glucan 1 protein, glycoside hydrolase family proteins, beta-glucanase, glucosidase, endo-N-acetyl-beta-D-glucosaminidase, chitinase, and invertase with log2fold change (LFC) of 64, 64, 64, 44.5, 44.5, 35.8, 33.2 and 26.9, respectively (Table 1). The various proteases that were found to be highly expressed in SSF were identified to include serine peptidase (LFC = 64), serine carboxypeptidase (LFC = 64), subtilisin-like serine protease (LFC = 47.4), etc.

Upregulation of B. bassiana SAN01 proteins under submerged fermentation (SF) conditions

In comparison, the percentage of upregulated proteins in the SF secretome amounted to 13 % of that in the SSF secretome. A significant portion of the 39 proteins found to be upregulated during SF, relative to SSF, were linked to chitin metabolism, such as chitinase and beta-N-acetylhexosaminidase (Table 2). Although some chitinolytic enzymes were also upregulated in SSF, the proportion of chitinolytic enzymes (in relation to the upregulated enzymes in each secretome) is significantly higher in SF than in SSF. However, unlike in SSF, where numerous lignocellulose-related enzymes were upregulated, only feruloyl esterase (LFC = 2.33) and a glycoside hydrolase (LFC = 3.13) were upregulated in SF (see Supplementary Table S2).

CAZyme annotation of B. bassiana SAN01 secretome

The CAZyme annotation of the Bb secretome under SF and SSF conditions revealed the presence of 147 and 236 carbohydrate-active enzymes, respectively (Table 3). Based on the number of the strains's CAZyme transcripts and the expressed proteins in this study, the translational efficiency of the fungus for CAZymes can be considered higher in the SSF secretome (64.3 %) compared to the

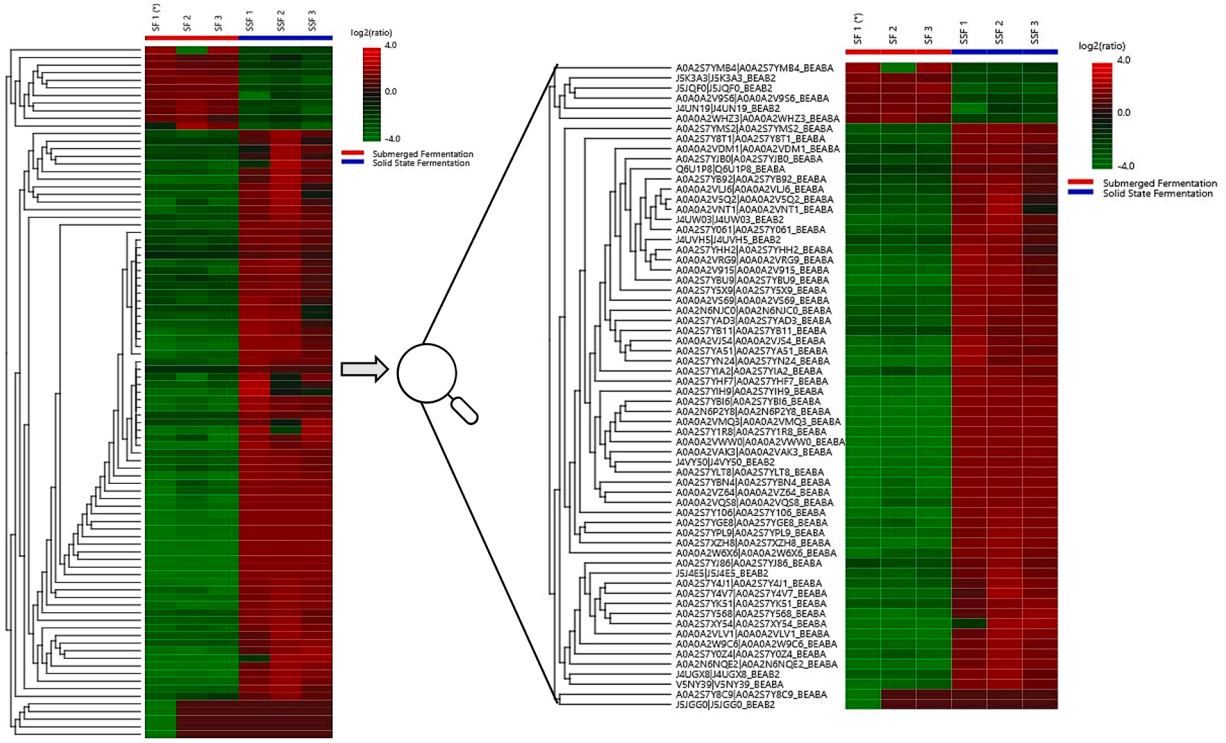

Fig. 2. Near here.

Table 1
Selected upregulated *B. bassiana* SAN01 proteins in the solid-state fermentation (SSF) secretome.

UniProt Accession	Protein	Log2Fold Change
J4WF58	Cell wall protein	64
J4UGX5	Phosphorylcholine phosphatase	64
A0A0A2VZ64	Glucan 1	64
A0A0A2VWW0	Cell wall mannoprotein	64
A0A2S7YBN4	Serine peptidase	64
A0A0A2VSW6	Cysteine-S-conjugate beta-lyase	64
A0A0A2VG56	Phosphorylcholine phosphatase	64
A0A2S7Y9S7	Pectate lyase superfamily protein domain-containing protein	64
J4VU46	Antifungal protein	64
A0A2S7YHF7	Phosphorylcholine phosphatase	64
J4W938	Glycoside hydrolase family	64
A0A2N6NAW9	Phosphorylcholine phosphatase	64
J4KNI1	Extracellular aldonolactonase	64
A0A2N6NQC5	Cell wall mannoprotein 1	64
A0A2N6NYD1	Putative extracellular serine carboxypeptidase	64
A0A2S7YAX8	Antifungal protein	64
J5JH96	Cell wall protein	64
A0A2S7Y1R8	6-phosphogluconolactonase	64
A0A0A2VQA0	Putative serine protease	64
A0A0A2VDW0	Alginate lyase 2 domain-containing protein	61
A0A2S7XY54	NLP/P60 protein	59.5
J4VQN5	Beta-hexosaminidase	56
A0A2S7YBU9	Peptidase M14 carboxypeptidase A domain-containing protein	50.4
A0A2N6NZ52	Metallocarboxypeptidase A-like protein	49
J4VWU1	Carboxypeptidase-like protein	49
J5JDQ6	Subtilisin-like serine protease	47.4
A0A0A2W1C4	Beta-glucanase	44.5
J4W3R0	Secreted glucosidase	44.5
A0A097F8P7	Glycoside hydrolase family 18	35.8
A0A2S7YNJ4	GH18 domain-containing protein	35.8
J5J285	Endo-N-acetyl-beta-D–glucosaminidase	35.8
J5JJE1	FAD binding domain-containing protein	33.8
A0A6H1NQ50	Chitinase	33.2
A0A0A2VE94	Putative glycosidase	28.9
A0A0A2W9C6	Invertase	26.9

Table 2
Selected upregulated *B. bassiana* SAN01 proteins in submerged fermentation (SF) secretome.

UniProt Accession	Protein	Log2Fold Change
J5K3A3	C6 transcription factor	11.11
A0A2S7YFK9	Carboxypeptidase C	10
J5JWX2	Beta-N-acetylhexosaminidase	7.14
J4KM52	Chitinase	6.67
A0A0A2VDY4	Antigenic thaumatin-like protein	4.76
A0A2S7Y6 × 7	Bys1 family protein	4.76
A0A0A2V9S6	Tripeptidyl-peptidase sed1	4.17
A0A2S7YBB3	Aorsin-like protein	4.17
A0A2N6N8K9	Peptidase S53 domain-containing protein	4.17
J4UIY3	Aspergillopepsin-2	4
A0A2N6N7T8	Peptidase A4 family protein	4
A0A2N6NR84	Chromo domain-containing protein	3.23
A0A097F8L3	Glycoside hydrolase	3.13
J4WDD0	Putative serine protease K12H4.7	2.44
A0A2N6NBQ5	Putative extracellular serine carboxypeptidase	2.44
A0A2S7XYP2	Putative aspartic protease	2.44
A0A0A2VBY0	Serine peptidase	2.44
A0A2N6P1H1	Feruloyl esterase	2.33
A0A0A2VKM0	Cutinase transcription factor 1 beta	2.33

SF (40.1 %). The CAZymes elucidated in the Bb secretome were grouped into 39 CAZyme families, which were subdivided into the 34 families of glycoside hydrolases (GHs), 3 families of auxiliary activities (AAs), and 2 families of carbohydrate esterases (CEs). However, it was observed that, in addition to the higher number of identified extracellular CAZymes in its SSF secretome, it also contained some CAZyme families that were lacking in the SF secretome, e.g., GH5_9, GH16_3, and GH16_18.

Table 3
Identified CAZyme in *B. bassiana* SAN01 submerged (SF) and solid-state fermentation (SSF) secretomes.

CAZy family	EC Number	Enzyme	Substrate	Copy number	
				SF	SSF
AA2	1.11.1.7	Lignin, manganese, & versatile peroxidases	Lignin	4	4
AA3_2	1.1.99.18	Cellobiose dehydrogenase	Cellobiose and other oligosaccharides	4	5
AA7	1.1.3-	Chitooligosaccharide, glucooligosaccharide and xylooligosaccharide oxidases	Oligosaccharide	7	7
CE1	3.1.1.72	Acetyl xylan esterase, feruloyl esterase	Xylan	2	2
CE5	3.1.1.74	Acetyl xylan esterase, cutinase	Xylan, cutinases	4	8
GH2	3.2.1.23 3.2.1.25	β-Galactosidase β-Mannosidase, α-L-Arabinofuranosidase, xylosidase	Lactose, mannan, xylan	2	6
GH3	3.2.1.21 3.2.1.37	β-Glucosidase β-xylosidase Endoglucanase, exoglucanase	Cellobiose, cellulose, xylobiose, xylan	9	20
GH5_9	3.2.1.4	β-Glucosidase, endoglucanase and exoglucanase	Cellulose, cellobiose	0	1
GH5_15	3.2.1.4	Endoglucanase	Cellulose	4	4
GH13_1	3.2.1.1	α-Amylase	Starch	4	4
GH13_8	3.2.1.133	Amylomaltase	Starch	1	1
GH15	3.2.1.3	Glucoamylase	Starch, glycogen, and related α-glucans	4	4
GH16_1	3.2.1-	β−1,3 glucanosyltransglucosidase, hyaluronoglucosaminidas, endo-β−1,3-glucanase / laminarinase	Laminarin, hyaluronic acid, beta-glucans	0	4
GH16_3	3.2.1-	β−1,3-glucanase, Endo-β−1,4-galactosidase, laminarinase	Laminarin, hyaluronic acid, beta-glucans	0	3
GH16_18	2.4.1-	β-transglycosidase Chitin- glucanosyltransferase	Chitin, laminarin, other polysaccharides	0	8
GH17	3.2.1.39, 3.2.1.58	Endoglucanase and exoglucanase	Cellulose	0	3
GH18	3.2.1.14	Chitinase	Chitin	26	31
GH20	3.2.1.52	β-N-Acetylhexosaminidase	Glycoproteins, glycosaminoglycans, and glycolipids	13	13
GH25	3.2.1.17	Lysozyme	Peptidoglycan	4	4
GH27	3.2.1.22	α-Galactosidase	Lactose and other galactose-containing oligosaccharides	8	8
GH31_1	3.2.1.20 3.2.1.22	α-Glucosidase α-Galactosidase	Starch and lactose	4	9
GH32	3.2.1.26 3.2.1.26	Fructan β-fructosidase Invertase	Sucrose and fructans	4	4
GH35	3.2.1.23	β-Galactosidase	Galactose-containing oligosaccharides	5	9
GH36	3.2.1.22	α-Galactosidase	Glycoside hydrolases, include α-galactosidases	0	4
GH38	3.2.1.-	Mannose	Glycoside hydrolases, primarily involved in α-mannosidase activity	0	1
GH43_34	3.2.1.55 3.2.1.37	Arabinofuranosidase β-Xylosidase	Arabinoxylans	4	4
GH47	3.2.1.113	α-Mannosidase	N-linked glycoproteins	1	5
GH54	3.2.1.99	Endo-1,5-α-arabinanase	Arabinan	4	4
GH55_2	3.2.1.58	Exo-β−1,3-glucanase	β−1,3-glucans	5	8
GH64	3.2.1.59	Endo-α−1,3-glucanase	Glucans	3	3
GH75	3.2.1.132	Chitosanase	Chitosan	4	4
GH76	3.2.1.20 3.2.1.101	Endo-α−1,6-mannanase α-glucosidase	Cellulose, mannan	0	4
GH79	3.2.1.31	β-Glucuronidase	β-glucuronides and glycosaminoglycans	3	7
GH84	3.2.1.52	β-N-Acetylglucosaminidase	Glycoproteins and glycolipids	4	4
GH89	3.2.1.50	Heparan sulfate	Glycoside hydrolases; α-N-acetylglucosaminidases	0	3
GH92	3.2.1.24	α-Mannosidase	Mannose-containing polysaccharides and glycoproteins	4	12
GH125	3.2.1.163	α−1,6-Mannosidase	Mannose-containing polysaccharides and glycoproteins	2	3
GH142	3.2.1.78	Endo-β−1,4-mannosidase	β−1,4-mannans	4	4
GH184	3.2.1.17	Muramidase	Peptidoglycan	0	4

Gene ontology enrichment analysis

The functional enrichment analysis of Bb secretome under both SF and SSF of lignocellulosic biomass classified the expressed proteins based on their biological processes (BP), molecular function (MF), cellular component (CC) as well as their protein domains (Fig. 3). The biological process functionalisation showed that the fungal secretome under both conditions was actively involved in polysaccharide deconstruction as the majority of the subcategories enumerated were either directly or indirectly linked to the utilisation of both simple and complex carbohydrates (Fig. 3a). For instance, carbohydrate metabolism as a subcategory of BP was shown to be prominent in both SF (12.8 %) and SSF (14.9 %), thus further highlighting the ability of Bb to utilise lignocellulosic biomass,

hence, justifying the high expression of carbohydrases. Other BP subcategories linked to carbohydrate utilisation include glycosaminoglycan metabolic process, glycoprotein catabolic process and polysaccharide catabolic process. However, proteolysis was observed as a more prominent BP in SF (29.9 %) than in SSF (17.2 %). The most prominent subcategories under the MF category were found to include serine-type endopeptidase, metal ion binding, chitin binding, chitinase, hydrolase, tripeptidyl-peptidase, GTPase and GTP binding activity (Fig. 3b).

Furthermore, annotation of Bb secretome into the CC category showed that extracellular activity was the most prominent subcategory under both conditions with percentages of 28.3 % and 19.0 % for SF and SSF, respectively, which is not unexpected as the secretome is principally made up of extracellular proteins (Fig. 3c). However, plasma membrane, fungal-type vacuole, cytosol and nucleus were also found to account for a significant portion of the CC category in both secretomes. The domain functional enrichment of the secretomes reveals the predominance of domains such as Pro-kuma_activ, RAS, RAB, LysM, WSC, UBQ, fCBD, Glyco_18, and Fn3_like domain (Fig. 3d). The Pro-kuma_activ domain was observed to be a major domain in both fungal secretomes; although it accounted for the highest proportion in SF (14.2 %), its proportion in the SSF secretome was significantly lower (7.5 %). The key domains linked to carbohydrate metabolism were identified to include WSC (a carbohydrate-binding domain), fCBD (a domain involved in cellulose binding) and Glyco_18 (found in hydrolytic enzymes with chitinase and endo-N-acetyl-beta-D-glucosaminidase activities). It is noteworthy that Tubulin_C and Tubulin domains were only found to occur in the SSF secretome; on the other hand, DUF663, KH, ZnF_C2HC, IENR1, IENR2, WH1, AARP2CN, ARID, GIYc, EFh, H3, CUE domains were observed to be present only in the SF secretome. A review of the InterPro and SMART databases revealed that these SF-exclusive domains are involved in various metabolic activities, including actin polymerisation, DNA repair, nucleic acid binding, metal ion binding, ribosome biogenesis, and signal transduction.

The Uniprot accession codes of the 341 differentially expressed proteins in Bb SAN01 were also observed to match 176 genes on the DAVID platform, which were associated with 7 enriched functional clusters. The DAVID GO enrichment analysis revealed that the molecular function (MF), biological process (BP), and cellular component (CC) categories accounted for 61.8 %, 50.9 %, and 41.8 %, respectively. Further elucidation showed that the biological functions of the proteins were mainly displayed in carbohydrate metabolism. In specific terms, six out of the 29 BP subcategories were directly linked to carbohydrate metabolism at p values ranging from 1.4×10^{-16} to 6.7×10^{-1}. These subcategories include carbohydrate metabolic process, carbohydrate derivative catabolic process,

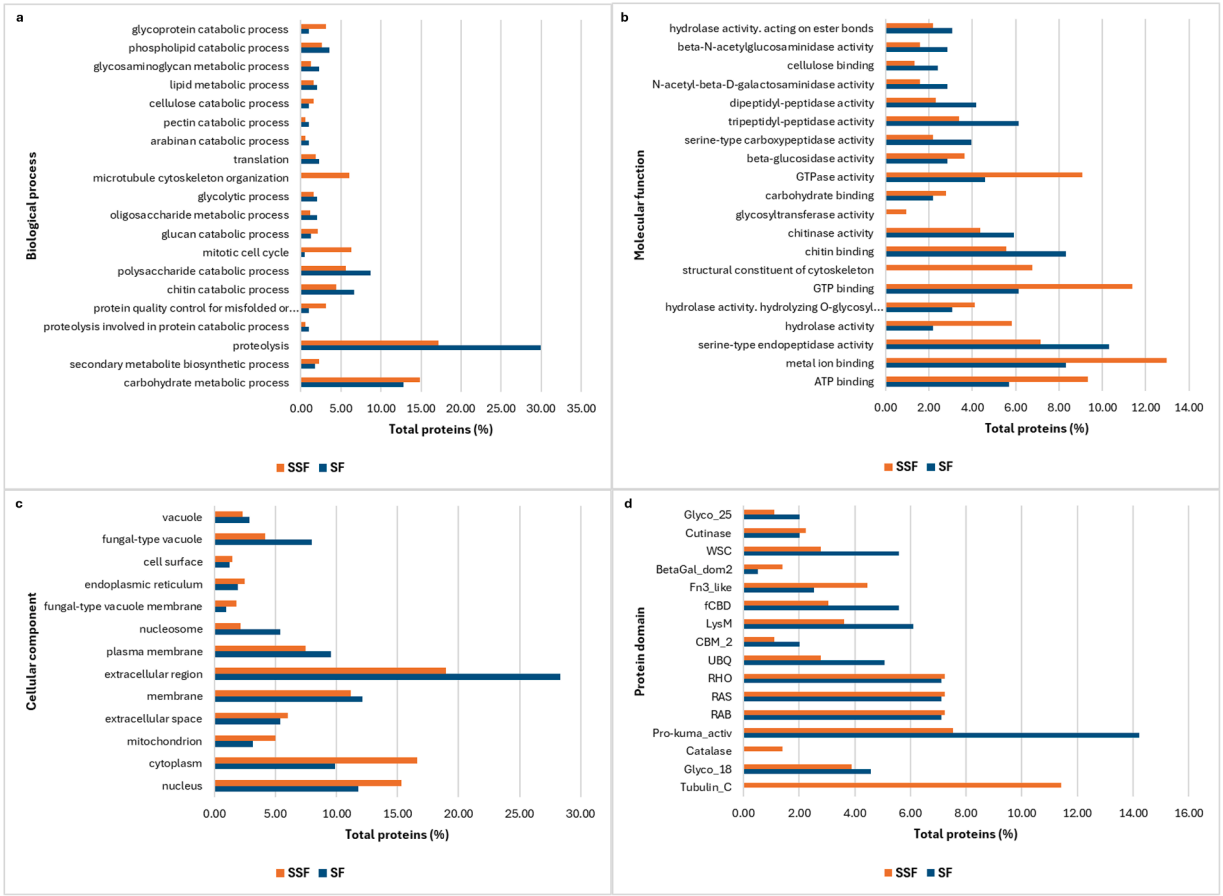

Fig. 3. Near here.

carbohydrate catabolic process and carbohydrate derivatisation processes. Other processes indirectly linked to carbohydrate metabolism include the glycoprotein catabolic process, the aminoglycan catabolic process, and aminoglycan metabolic process. The predominance of hydrolytic catalysis, especially carbohydrate and protein hydrolysis, was also strongly demonstrated in the MF annotation, with 21 out of the 23 molecular functions directly related to enzymatic activities.

Discussion

The dynamics of lignocellulose degradation and carbohydrase production by *B. bassiana* SAN01, an entomopathogenic fungal endophyte, were elucidated in this study. The significant potential of this FF in utilising lignocellulosic biomass as a cost-effective bioresource for important bioprocesses has been previously highlighted by the characterisation of its genome [7] as well as its transcriptome under different conditions [6]. The genome of the fungus was predicted to encode 10,366 protein genes which include approximately 145 carbohydrate-active enzymes and 314 proteases [7]. Notably, higher production of extracellular proteins by the fungus was observed under SSF relative to SF, and this might be because the conditions provided by SSF are more closely related to its natural biological condition as a filamentous fungus, which is typically adapted to grow on solid surfaces and relatively low water activity [12]. This hypothesis has been corroborated by previous studies that have shown higher extracellular protein secretion in FF during SSF relative to SF [20,21]. For example, the amounts of protein in SSF in comparison to SF were recorded to be 30-fold in *Neurospora sitophila* [21] and 2-fold in *Phanerochaete chrysosporium* [20]. Expectedly, proteins belonging to the carbohydrases group were observed to be copiously secreted under both SSF and SF which is due to the lignocellulosic nature of the substrate being metabolised by the fungi. The ability of FF and other microbes has been noted to be influenced by their growth substrate. Recently, *Parascedosporium putredinis* was demonstrated to produce numerous carbohydrases when grown on various lignocellulosic biomasses, including rice straw, sugarcane bagasse, wheat bran, wheat straw, and oil palm fruit bunches [22]. However, despite the fact that *B. bassiana* SAN01 has been established previously to possess the potential to secrete diverse carbohydrases [6], the high expression of these enzymes remains remarkable, thus highlighting its metabolic plasticity and versatility. The high number of proteases recorded in both secretomes is in agreement with previous studies as proteases have long been identified as critical enzymes required for the fungus to exhibit its well-known entomopathogenic role in nature [15]. Notably, the correlation within group recorded in this study showed a high degree of similarity among biological replicates within each fermentation group, establishing the reliability of the proteomic data and the consistency of the fermentation processes. Hence, the differential extracellular expression observed can be attributed to actual biological responses to the distinct fermentation environments as opposed to experimental variability.

The SignalP prediction of the Bb proteins categorised the secretome based on the presence of signal peptides. These signal peptides are peptide sequences that direct proteins to be extracellularly secreted or to be localised in specific compartments within the cell. The signal peptides, typically found in the protein's N-terminus, facilitate the passage of proteins across biological membranes via the general secretory (Sec) pathway, a ubiquitous cellular process critical in industrial applications [23]. However, it has been hypothesised that some proteins might still be extracellular despite the lack of the signal peptide; these categories of proteins might have employed alternate secretion pathways such as secretory lysosome pathways, exosomes, and autophagy-related mechanisms, to mention a few [24]. Besides supporting the authenticity of extracellular localisation as recorded in LC-MS/MS, the prevalence of signal peptides suggests that Bb has a robust secretory system, capable of efficiently exporting large quantities of proteins to the extracellular medium. This property makes the FF a promising host for the production of recombinant enzymes and other industrial proteins, as well as large-scale production in bioreactors or fermentation systems [25]. The THMMM analysis of the Bb secretome computed the likelihood of each protein being embedded in the cellular membrane and not extracellularly released. It is believed that the transportation function of these proteins demands that they interact significantly with the membrane, hence, the presence of transmembrane helices. The THMMM results, which showed that only a small proportion of Bb proteins had low propensity of being transmembrane localised, further support the signal peptide predictions, where the majority of secreted proteins possessed N-terminal signal sequences characteristic of the classical ER–Golgi secretion pathway. Together, these results signify the biological authenticity of the secretome preparation, minimising the likelihood of intracellular protein contamination and underscoring Bb's strong protein secretory capacity. However, some non-transport proteins, such as carboxypeptidase, endo-beta-N-acetylglucosaminidase F2 protein, metal tolerance protein, threonylcarbamoyl-AMP synthase, and WSC domain-containing protein, were predicted with the transmembrane helices. The endo-beta-N-acetylglucosaminidase F2 protein from the fungus, which has seven predicted transmembrane helices, indicates that it is a membrane protein with multiple transmembrane regions. Endo-beta-N-acetylglucosaminidases are versatile and conserved enzymes that catalyse the metabolism of N-linked glycans, and they have been demonstrated to carry out their hydrolytic activity in both the cytoplasm and the extracellular space [26].

PCA revealed a distinct separation between the secretomes obtained from SF and SSF, accounting for close to 80 % of the total variance in the dataset. As also demonstrated earlier through correlation analysis, the distinct clustering of biological replicates within each fermentation condition indicates significant experimental reproducibility and substantial biological divergence between the two fermentations. The clear separation along the principal components also indicates that Bb exhibits distinct protein expression profiles under each condition, reflecting specific physiological adaptations to the fermentation environment. Similarly, the heatmap-based clustering signifies Bb's ability to dynamically reprogram its proteome in response to fermentation mode, with the prominence of extracellular and stress-related proteins under SSF highlighting the FF's adaptive strategies for solid substrate colonisation [27].

Comparative proteomic analysis revealed that the SSF secretome displayed a substantial upregulation of 301 proteins (~25 % of the total expressed proteins), compared to only 39 proteins in the SF secretome, which accounts for approximately 13 % of the SSF upregulated proteins. This disparity highlights the robust metabolic activation and extracellular enzyme secretion induced in Bb during SSF, aligning with recent studies that have highlighted the enhanced production of extracellular enzymes in FF under similar

conditions. For instance, the concentration and diversity of extracellular proteins in *Aspergillus brasiliensis* were approximately 4.6- and 3.9-fold in SSF compared to SF [13]. Recently, 14 proteins were observed to be upregulated in SSF compared to SF during the cultivation of *Penicillium janthinellum* on rice straw [28]. A prominent feature of the SSF secretome was the substantial upregulation of enzymes involved in lignocellulose degradation, including pectate lyase, glucanases, glycoside hydrolases, β-glucanase, glucosidase, chitinase, endo-N-acetyl-β-D-glucosaminidase, and invertase. These enzymes are pivotal for lignocellulose deconstruction in plant biomass, further demonstrating that SSF conditions induce Bb to enhance its enzymatic arsenal for efficient nutrient acquisition from solid substrates. This is further supported by findings from our previous study, where transcriptomic analysis of *B. bassiana* during wheat bran metabolism revealed that genes encoding carbohydrases such as pectate lyase and proteases such as serine protease, were among the top-most upregulated genes [6]. The increased activity of some glycosyl hydrolases under SSF in comparison to SF has also been demonstrated quantitatively in various studies. For instance, the production levels of β-glucosidase, endoglucanase and esterase activities were significantly higher in *Phanerochaete chrysosporium* during SSF relative to SF [20]. Although there is no definitive explanation for the high expression in SSF, it was previously posited that catabolite repression is typically limited in SSF, which in turn facilitates hyphal branching and enhances the secretion of diverse proteins, including extracellular enzymes [13]. Additionally, it has been demonstrated that the abundance of hemicellulolytic enzymes and other essential accessory enzymes in SSF results in higher biomass hydrolysis efficiency [28].

Interestingly, the significant upregulation of some proteolytic enzymes may also be related to their involvement in plant biomass degradation, as was previously highlighted by Wang, Hart [29]. On the other hand, the SF secretome exhibited a higher proportion of chitinolytic enzymes among its upregulated proteins, thus suggesting that these conditions may preferentially activate pathways associated with chitin metabolism. The limited upregulation of lignocellulose-degrading enzymes in SF also supports the fact that submerged conditions may not strongly stimulate complex polysaccharide-degrading pathways. Chitinases are naturally one of the most prominent enzymes produced by Bb in nature due to their biological role as entomopathogens. Hence, Bb chitinase production has been demonstrated severally under SSF and SF conditions [30,31]. However, besides its key role in entomopathogenic virulence, chitinases have also been noted to play active roles in fungal growth, morphogenesis, and autolysis, a natural self-degradation process in FF [32]. It is hypothesised that the higher chitinase expression in SF may be attributed to the fact that the liquid environment of SF more closely mimics the typical conditions in the insect hemolymph, where Bb interacts with its pests and degrades chitin-rich cuticles. This hypothesis may also explain the upregulation of some proteases in SF (carboxypeptidase C, LFC = 10; tripeptidyl-peptidase sed1, LFC = 4.12; aorsin-like protein = 4.12; peptidase S53 domain-containing protein LFC = 4.12, etc.) as proteases are also principal virulence factors of the fungus. In addition, it is also posited that the submerged condition might have elicited higher stress due to osmotic pressure, which might have further induced chitinase production as a survival mechanism. Supporting this hypothesis is the finding that other stress tolerance proteins were upregulated in the SF conditions, for example, the antigenic thaumatin-like protein (LFC = 4.76).

FF generally secrete an array of carbohydrate-active enzymes (CAZymes), otherwise referred to as glycosyl hydrolases, which facilitate the efficient degradation of the available biomass in their habitat and may reflect their specialised habitat-related substrate utilisation. Furthermore, it has been proposed that profiling these groups of enzymes in an organism could enhance its industrial applicability in biomass utilisation and organic pollutant degradation [33]. The 147 (SF) and 236 (SSF) CAZymes annotated in the fungus align with our previous study, where the fungus was demonstrated to contain 367 CAZyme-encoding transcripts while utilising the same lignocellulosic biomass for its growth [6]. Although the relationship between transcripts and protein levels is not always straightforward, however, the number of transcripts is typically higher than the number of expressed proteins due to various factors such as alternative splicing, post-transcriptional regulation, mRNA stability and degradation, non-coding RNAs, which are all related to gene expression and regulation [34]. Furthermore, there is a very high likelihood that, besides the extracellular CAZymes annotated in this study, there are other CAZymes located in the cytoplasmic and periplasmic space, which may also account for some of the transcript-protein discrepancies. In addition, regarding its extracellular CAZyme profile, Bb SAN 01 can also be considered to compete favourably when compared with the secretome of some other filamentous fungi under closely related conditions, e.g., *Phanerochaete chrysosporium* [20]. For instance, *P. chrysosporium*, a typical white-rot fungus, was demonstrated to secrete 110 and 64 extracellular carbohydrate-active enzymes under SSF and SF conditions, respectively [20]. This further signifies the secretome complexity of Bb under SSF conditions, as previously surmised in a similar study on *Aspergillus brasiliensis* [13]. Findings from the current study also show that Bb contains various CAZymes that can digest a wide spectrum of polysaccharides, including cellulose, hemicellulose, lignin, starch, as well as chitin, peptidoglycans and cutin, to mention a few. This high secretome complexity under SSF reflects its significant advantage for the industrial production of CAZymes, as the biomass can serve as both the solid support and the nutrient source for the fungus, leading to immense economic and environmental benefits [27]. For instance, the GH3 CAZymes, namely β-glucosidase, β-xylosidase, endoglucanase, and exoglucanase, are cellulolytic enzymes that efficiently hydrolyse lignocellulosic biomass, a key step in biofuel production and biomass valorisation [35]. Similarly, amylases, are important enzymes in food processing (dough quality enhancer, starch conversion to fermentable sugars for alcohol production), textile (fabric desizing), detergents, to mention a few [36].

The classification of the secretome into biological processes (BP), molecular functions (MF), cellular components (CC), and protein domains provides deep insight into the biological roles and potential applications of the proteins under both fermentation modes. It also establishes the fact that both the SF and SSF secretomes of Bb are actively involved in carbohydrate metabolism and polysaccharide degradation, which is consistent with the upregulation of carbohydrases observed in the differential expression analysis. Notably, the enumeration of serine-type endopeptidase and tripeptidyl-peptidase as key MF is also in accordance with the previous observation about proteolysis being a major BP under both SF and SSF. Furthermore, the prominence of chitin-binding and chitinase activity in the MP suggests that a significant proportion of its carbohydrate metabolism is chitin-related; this is not a far-fetched theory, judging from the fact that chitinases are among the principal enzymes of entomopathogenic fungi [30,31]. Interestingly, metal ion

binding activity was recorded as a key MF under both SF (8.3 %) and SSF (13 %), it is posited that this may be due to the cofactor requirement of the various enzymes actively present in the secretomes. For instance, approximately one-third of proteins from *Paracoccidioides* fungi contained at least one metal cofactor, with Cu-, Fe-, and Zn-binding proteins representing ~7 % of their total proteins [37]. The metal-chelating activity may also suggest the potential biotechnological application of Bb SAN01 in the bioremediation of heavy-metal-contaminated environments as highlighted previously [38]. In the recent study, Bb was demonstrated to bioaccumulate heavy metals (Cu(II), Ni(II), Cd(II), Zn(II), Cr(VI) and Pb(II)) as well as dyes (indanthrene blue, reactive remazol red, yellow 3RS, and vat novatic grey) [39]. In this study, other MF highlighted for Bb include carbohydrate:proton symporter activity, fatty acid synthase activity, protein-lysine N-methyltransferase activity, and peroxidase activity, among others. Pro-kuma_activ is an α and β sandwich folded propeptide prevalent in various proteases and it has been identified to play critical functions in the activation and regulation of enzymes involved in microbial pathogenicity, survival, and adaptation [40]. While the LysM domain is found in most extracellular proteins, the UBQ and fn3 domains are implicated in general cellular functions such as cell adhesion, cell migration and cell growth [41].

Conclusion

The LC-MS/MS based proteomic approach employed in this study complements existing knowledge of the genome and transcriptome of the entomopathogenic fungal endophyte, *B. bassiana*. Findings show the dynamic biomass degrading machinery of Bb, highlighting the higher diversity and expression levels of extracellular proteins in the SSF secretome compared to the SF. The total protein expression profile under both cultivation methods and the differentially expressed proteins indicate increased secretion of polysaccharide degrading enzymes under SSF conditions, which suggests more efficient biomass deconstruction and lower catabolite repression. Additionally, the higher expression of structural proteins under SSF indicates increased hyphal activity in the solid matrix relative to the liquid environment. However, the lower difference observed in the expression and activities of proteolytic enzymes suggests that the fungi conserved the semblance of their typical entomopathogenic role under both conditions. Furthermore, various hypothetical proteins were detected in the secretomes of the fungus, potentially indicating the involvement of a wide range of biochemical pathways in Bb for biomass degradation that have not yet been elucidated. Like every study, some limitations were also identified in this study. For instance, sampling the secretomes at different points- as opposed to only at the end of the fermentation- would have provided greater insights into the dynamism of the fungus as proteins are continually being turned over with time. Similarly, the concentration of the secretome prior to LC-MS analysis may have also caused significant losses of low-molecular-weight compounds, such as mediators or cofactors. Despite all this, the study has further revealed that Bb SAN01 has considerable potential in lignocellulosic biomass deconstruction and in the production of lignocellulolytic enzymes. By revealing an indigenous fungal resource with industrial enzyme potential, the work supports the development of Africa's circular bioeconomy, informs policy directions for waste valorisation, and strengthens local capacity for climate-resilient, resource-efficient bioprocessing. However, it is recommended that further investigation be conducted, including detailed characterisation of individual enzymes, evaluation of other biomass, optimisation of cultivation conditions, as well as strain improvement.

Availability of data and material

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2025.e03080.

References

[1] M. Gandía, S. Garrigues, Filamentous fungi as excellent industrial strains: development and applications, J. Fungi 10 (8) (2024) 541, https://doi.org/10.3390/jof10080541.
[2] Z. Merényi, et al., Genomes of fungi and relatives reveal delayed loss of ancestral gene families and evolution of key fungal traits, Nat. Ecol. Evol. 7 (8) (2023) 1221–1231, https://doi.org/10.1038/s41559-023-02095-9.
[3] P. Umashankar, Y. Nygård, Filamentous fungi as emerging cell factories for the production of aromatic compounds, Fungal Biol. Biotechnol. 11 (1) (2024) 19, https://doi.org/10.1186/s40694-024-00188-z.
[4] D. Troiano, V. Orsat, M.J. Dumont, Status of filamentous fungi in integrated biorefineries, Renew. Sustain. Energy Rev. 117 (2020) 109472, https://doi.org/10.1016/j.rser.2019.109472.
[5] E.A. Alves, et al., Process development to obtain a cocktail containing cell-wall degrading enzymes with insecticidal activity from *Beauveria bassiana*, Biochem. Eng. J 156 (2020) 107484, https://doi.org/10.1016/j.bej.2019.107484.
[6] A. Amobonye, et al., Transcriptomic profiling of *Beauveria bassiana* SAN01, an endophytic fungal entomopathogen, for the production of lignocellulosic enzymes, Biocatal. Agric. Biotechnol. 54 (2023) 102918, https://doi.org/10.1016/j.bcab.2023.102918.
[7] G. Xiao, et al., Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*, Sci. Rep. 2 (1) (2012) 483, https://doi.org/10.1038/srep00483.
[8] B. Amer, E.E.K. Baidoo, Omics-driven biotechnology for industrial applications, Front. Bioeng. Biotechnol. 9 (2021) 2021, https://doi.org/10.3389/fbioe.2021.613307.
[9] C. Du, G.P. van Wezel, Mining for microbial gems: integrating proteomics in the postgenomic natural product discovery pipeline, Proteomics 18 (18) (2018) 1700332, https://doi.org/10.1002/pmic.201700332.
[10] N. Joshi, et al., Bioprospecting CAZymes repertoire of *Aspergillus fumigatus* for eco-friendly value-added transformations of agro-forest biomass, Biotechnol. Biofuels Bioprod. 17 (1) (2024) 3, https://doi.org/10.1186/s13068-023-02453-6.
[11] M.A. de Assis, et al., A multiomics perspective on plant cell wall-degrading enzyme production: insights from the unexploited fungus *trichoderma erinaceum*, J. Fungi 10 (6) (2024) 407, https://doi.org/10.3390/jof10060407.
[12] M. Vassileva, et al., Effect of the mode of fermentation on the behavior of *penicillium bilaiae* in conditions of abiotic stress, Microorganisms. 11 (4) (2023) 1064, https://doi.org/10.3390/microorganisms11041064.
[13] D. Salgado-Bautista, et al., Solid-state fermentation increases secretome complexity in *Aspergillus brasiliensis*, Fungal. Biol. 124 (8) (2020) 723–734, https://doi.org/10.1016/j.funbio.2020.04.006.
[14] K. Oda, et al., Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions, Appl. Env. Microbiol. 72 (5) (2006) 3448–3457, https://doi.org/10.1128/AEM.72.5.3448-3457.2006.
[15] Y. Bian, C. Gao, B. Kuster, On the potential of micro-flow LC-MS/MS in proteomics, Expert Rev. Proteom. 19 (3) (2022) 153–164, https://doi.org/10.1080/14789450.2022.2134780.
[16] O.H. Lowry, et al., Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1) (1951) 265–275.
[17] P. Czaplewska, et al., Trial proteomic qualitative and quantitative analysis of the protein matrix of submandibular sialoliths, Molecules 26 (21) (2021) 6725, https://doi.org/10.3390/molecules26216725.
[18] M.P. Tavares, et al., Comparative analysis of *Chrysoporthe cubensis* exoproteomes and their specificity for saccharification of sugarcane bagasse, Enzyme Microb. Technol. 173 (2024) 110365, https://doi.org/10.1016/j.enzmictec.2023.110365.
[19] P.K. Busk, et al., Homology to peptide pattern for annotation of carbohydrate-active enzymes and prediction of function, BMC Bioinform. 18 (1) (2017), 214 10.1186/s12859-017-1625-9.
[20] J. Liu, et al., Comparative characterization of extracellular enzymes secreted by *phanerochaete chrysosporium* during solid-state and submerged fermentation, Int. J. Biol. Macromol. 152 (2020) 288–294, https://doi.org/10.1016/j.ijbiomac.2020.02.256.
[21] Y. Li, X. Peng, H. Chen, Comparative characterization of proteins secreted by *Neurospora sitophila* in solid-state and submerged fermentation, J. Biosci. Bioeng. 116 (4) (2013) 493–498, https://doi.org/10.1016/j.jbiosc.2013.04.001.
[22] J.R. Scott Conor, et al., *Parascedosporium putredinis* NO1 tailors its secretome for different lignocellulosic substrates, Microbiol. Spectr. 12 (7) (2024) e03943, https://doi.org/10.1128/spectrum.03943-23. –03923.
[23] S. Grasso, et al., Signal peptide efficiency: from high-throughput data to prediction and explanation, ACS Synth. Biol. 12 (2) (2023) 390–404, https://doi.org/10.1021/acssynbio.2c00328.
[24] N. Miura, M. Ueda, Evaluation of unconventional protein secretion by *saccharomyces cerevisiae* and other fungi, Cells 7 (9) (2018) 128, https://doi.org/10.3390/cells7090128.
[25] K. Wei, et al., Exploring secretory signal sequences useful in excreting recombinant proteins in *Beauveria bassiana* as biocontrol fungus, Arch. Microbiol. 206 (12) (2024) 463, https://doi.org/10.1007/s00203-024-04190-2.
[26] G. Tzelepis, M. Karlsson, T. Suzuki, Deglycosylating enzymes acting on N-glycans in fungi: insights from a genome survey, Biochim. Biophys. Acta (BBA) - Gen. Subj. 1861 (2017) 2551–2558, https://doi.org/10.1016/j.bbagen.2017.08.022.
[27] P. Leite, et al., Recent advances in production of lignocellulolytic enzymes by solid-state fermentation of agro-industrial wastes, Curr. Opin. Green Sustain. Chem. 27 (2021) 100407, https://doi.org/10.1016/j.cogsc.2020.100407.
[28] M. Sankar, et al., Comparison of the solid-state and submerged fermentation derived secretomes of hyper-cellulolytic *penicillium janthinellum* NCIM 1366 reveals the changes responsible for differences in hydrolytic performance, Bioresour. Technol. 371 (2023) 128602, https://doi.org/10.1016/j.biortech.2023.128602.
[29] H. Wang, D.J. Hart, Y. An, Functional metagenomic technologies for the discovery of novel enzymes for biomass degradation and biofuel production, BioEnergy Res. 12 (3) (2019) 457–470, https://doi.org/10.1007/s12155-019-10005-w.
[30] L.B.P. Figueroa, et al., Enzyme production by the entomopathogenic fungi *beauveria bassiana* and *metarhizium anisopliae* and their application in the control of nematodes (*Haemonchus* spp. and *Meloidogyne incognita*) in vitro, J. Nat. Pestic. Res. 8 (2024) 100077, https://doi.org/10.1016/j.napere.2024.100077.
[31] R.R. Fantatto, et al., Determination of the best conditions to produce the Enzyme Chitinase by the Fungus Beauveria bassiana Proceedings 103 (1) (2024) 26.
[32] D. Thakur, et al., An overview of fungal chitinases and their potential applications, Protoplasma 260 (4) (2023) 1031–1046, https://doi.org/10.1007/s00709-023-01839-5.
[33] T. Sun, et al., Whole genome sequencing and annotation of *Naematelia aurantialba* (Basidiomycota, edible-medicinal fungi), J. Fungi. 8 (1) (2022) 6, https://doi.org/10.3390/jof8010006.
[34] G.T.P. Brancini, et al., Combining transcriptomics and proteomics reveals potential post-transcriptional control of gene expression after light exposure in *Metarhizium acridum*, G3 Genes Genomes Genet. 9 (9) (2019) 2951–2961, https://doi.org/10.1534/g3.119.400430.
[35] S. Kant, et al., Fungal cellulases: a comprehensive review, Nucleus (2024) 1–17, https://doi.org/10.1007/s13237-024-00501-6.
[36] R. Paul, J. Gogoi, N. Talukdar, Amylase from fruit and vegetable waste: its immobilization and application in the new trend—A review, Biotechnol. Appl. Biochem. (2025), https://doi.org/10.1002/bab.70012.
[37] G.B. Tristão, et al., Predicting copper-, iron-, and zinc-binding proteins in pathogenic species of the *Paracoccidioides* genus, Front. Microbiol. (2015) 2014, https://doi.org/10.3389/fmicb.2014.00761, 5 –.
[38] X. Zhang, et al., Genetic response analysis of *Beauveria bassiana* Z1 under high concentration Cd(II) stress, J. Hazard Mater. 464 (2024) 132984, https://doi.org/10.1016/j.jhazmat.2023.132984.

[39] D. Gola, A. Malik, M. Namburath, S.Z. Ahammad, Removal of industrial dyes and heavy metals by *Beauveria bassiana*: FTIR, SEM, TEM and AFM investigations with Pb (II), Environ. Sci. Pollut. Res. 25 (21) (2018) 20486–20496.
[40] S. Patel, Pathogenicity-associated protein domains: the fiercely-conserved evolutionary signatures, Gene Rep. 7 (2017) 127–141, https://doi.org/10.1016/j.genrep.2017.04.004.
[41] K. Škrlec, B. Štrukelj, A. Berlec, Non-immunoglobulin scaffolds: a focus on their targets, Trends Biotechnol. 33 (7) (2015) 408–418, https://doi.org/10.1016/j.tibtech.2015.03.012.