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Proteome Profile of *Aspergillus terreus* Conidia at Germinating Stage: Identification of Probable Virulent Factors and Enzymes from Mycotoxin Pathways

Raman Thakur · Jata Shankar

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Abstract *Aspergillus terreus* is an emerging opportunistic fungal pathogen that causes invasive aspergillosis in immunocompromised individuals. The main risk group of individuals for this organism is leukopenic patients, individuals having cancers, bone marrow transplant persons and those who have immunological disorders. The lack of early diagnostic marker for *A. terreus* and intrinsic resistance to Amphotericin B, further limits the successful therapy of *A. terreus*-associated infections. The germination of inhaled conidia is the key step to establish successful invasion in host tissues or organs. Thus, profiling of expressed proteins during germination of conidia not only shed light on proteins that are involved in invasion or virulence but may also provide early diagnostic markers. We used nanoLC-Q-TOF to study the proteome of germinating conidia (at 16 h time points) of *A. terreus*. We observed expression of 373 proteins in germinating conidia of *A. terreus*. A total of 74 proteins were uncharacterized in the database. The expressed proteins were associated with various processes like cell wall modulation, virulence factors and secondary metabolite biosynthesis. The most abundant proteins were associated with protein biosynthesis, carbohydrate metabolism and unknown functions. Among virulent proteins, mitogen-activated protein kinase (hog1) and mitogen-activated protein kinase (mpkC) are key virulent proteins observed in our study. We observed 7 enzymes from terretonin and 10 enzymes from geodin mycotoxin biosynthesis pathway. Interestingly, we observed expression of terrelysin protein, associated with blood cell lysis. Quantitative RT-PCR analysis showed 26-fold increase in transcripts encoding for dihydrogeodin oxidase and 885-fold for terrelysin gene in germinating conidia in comparison to conidia. Further, we propose that terrelysin protein and secondary metabolite such as geodin could be explored as diagnostic marker for *A. terreus*-associated infections.

Keywords *Aspergillus terreus* · Germination · Conidia · Mitogen-activated protein kinase · Terrelysin · Geodin

Introduction

Aspergillus terreus is a fungal pathogen present in air and soil [1]. It causes invasive infection in immunocompromised patients, and in recent years, *A. terreus*

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R. Thakur · J. Shankar (✉)
Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat Solan, Himachal Pradesh 173234, India
e-mail: jata.shankar@juit.ac.in; jata_s@yahoo.com

has gain importance among invasive fungal pathogens [2, 3]. The frequency of *A. terreus* associated with invasive aspergillosis varies from 1 to 30% where as the cases of invasive bronchopulmonary aspergillosis (IBA) reached to 3–12.5% with mortality rate of 98% [4, 5]. With the increased incidence of *A. terreus* infection and resistance to antifungal therapy especially to Amphotericin B (AMB), and a higher rate of invasive aspergillosis associated mortality (51 vs. 30%) compared with non-terreus species of *Aspergillus* require attention [6]. Due to the small size of *A. terreus* conidia and ubiquitous in nature, every day thousands of these conidia inhaled by humans that may settle to the lower respiratory tract [5]. These conidia may persist in immune cells such as macrophages for long time and also act as transporter of *A. terreus* conidia to secondary organs [4]. Inhaled conidia in immunocompetent individuals are cleared by immune cells, but if the immune system of individual is impaired or severely suppressed such as those in individuals that undergo bone marrow transplant, conidia start to germinate and form hyphae to cause invasive or systemic infections [4, 6, 7]. Thus, the transition of conidia to the germ tube followed by mycelia is a critical step in establishing systemic infection and host's mileu can influence the transition [8, 9]. Despite the importance of the early morphological transition, the mechanism of germination of *A. terreus* conidia is not well understood. Necessary knowledge on mechanism on transition may expedite the development of better diagnostic and therapeutic strategies to combat invasive aspergillosis caused by *A. terreus*.

With the availability of sequenced genomes of various *Aspergillus* species, high throughput approaches such as transcriptomic and proteomic were used to identify pathways, new diagnostic and therapeutic marker for *Aspergillus*-associated infections [10, 11]. Further, proteomics studies have been conducted on different morphological stages of *A. fumigatus* and *A. flavus* to decipher proteins involved in the invasion of host tissues, virulence or probable drug targets [12–16]. However, there are limited proteomic studies on *A. terreus* to decode proteins or pathways involve in the morphological transition from conidia to germ tube to hyphae/mycelia [17–19]. Also, due to emergence of resistance isolates of *A. terreus* against antifungal agents, it is important to study the proteins/pathways of *A. terreus* that are expressed during morphological transitions. Thus, the present study was carried out to profile proteins that are involved in germination of conidia of *A. terreus*. The overall approach of current work is given in Fig. 1. We observed expression of various proteins that are involved in cell wall modulation, and identification of probable virulence factor and enzymes involved in biosynthesis of mycotoxins.

Materials and Methods

Fungal Strain and Growth Condition

Aspergillus terreus strain (NCCPF 860035) isolated from induced sputum was acquired from National Culture Collection of Pathogenic Fungi (NCCPF), PGIMER Chandigarh, India for the current study. *Aspergillus terreus* strain was cultured on Sabouraud dextrose agar media (HiMedia, India) for the period of 1 week at 37 °C to produce conidia. Conidia were harvested by using phosphate buffer saline (pH 7.4) having 0.05% tween-20 (PBST), and the conidial suspension was filtered. Filtered sample was centrifuged at 1800 g for 5 min and further washed with ice-cold PBS 4 times [20]. The conidial suspension purity was evaluated using light microscopy. The obtained conidia were snap chilled with liquid nitrogen and stored at −80 °C for protein extraction [15].

Identification of *Aspergillus terreus* Morphotypes

Conidia were inoculated into Dulbecco's Modified Eagle Medium (DMEM pH 7.4, HiMedia, India) supplemented with 10% fetal bovine serum (FBS, HiMedia, India). Before inoculation into DMEM medium, conidial viability was checked on SDA media plates and CFU/ml was calculated to count viable conidia. DMEM medium inoculated with *A. terreus* conidia was incubated at 37 °C with continuous shaking (120 rpm). At every an hour interval, the fungal development was monitored under a light microscope (Magnus MPS-USB, Olympus, India) to identifying different morphotypes of *A. terreus*. Morphotypes of *A. terreus* were observed as resting conidia, swollen conidia, germinating conidia and hyphae. The percentage of conidial germination was determined by examining the conidial growth. In a

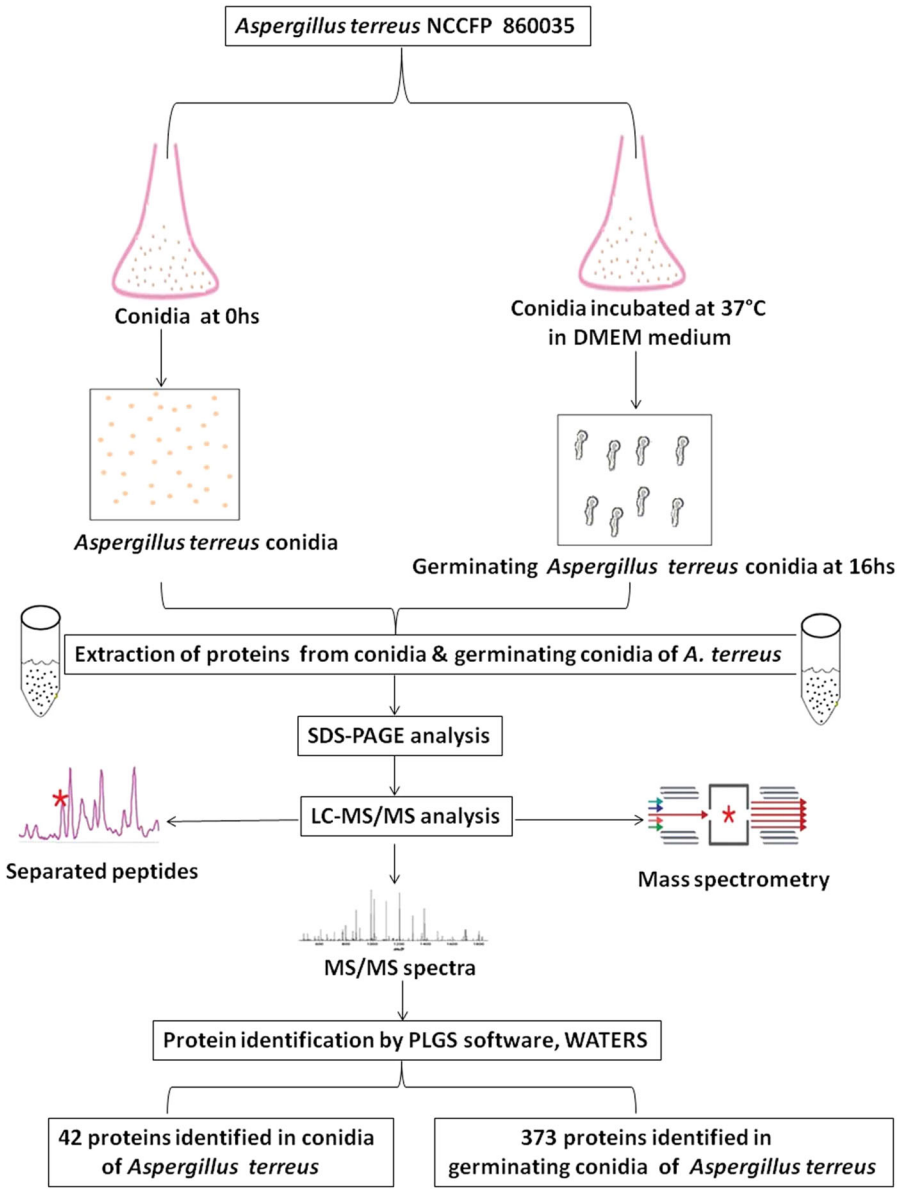

Fig. 1 Overall strategy followed to identify proteins in *Aspergillus terreus* conidia and in germination of conidia

triplicate count of conidia, more than 90% conidia were at germinating stage.

Preparation of Germinating Conidia

Aspergillus terreus conidia (1×10^6 conidia/ml) were inoculated into 100 ml of DMEM medium containing 10% FBS in culture flasks at 37 °C with continuous shaking (120 rpm). The conidial growth was monitored under a light microscope. After achieving more than 90% germ tube formation, samples were centrifuged at 15,000 rpm for 10 min at 4 °C to separated supernatant. These samples were washed with ice-cold PBS (pH 7.4) 4 times to remove the traces of media. Germ tube samples were snap chilled in liquid nitrogen and stored at −80 °C for protein extraction.

Extraction of Proteins from Conidia and Germinating Conidia of *Aspergillus terreus*

Cells were grounded in liquid nitrogen using pestle and mortar to achieve fine conidial lysate powder [12]. Total protein was extracted for conidial or germ tube

lysated at 4 °C using 50 mM sodium phosphate lysis buffer pH 7.4 (5 mL/mg) containing 0.2 mM DTT, 2 mM EDTA and 1 mM PMSF with constant stirring at 4 °C for 3 h. Cell lysate of conidia or germinating conidia were transferred to a centrifuge tube and centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant was transferred to centrifuge tubes, and proteins were precipitated with the help of 5% trichloroacetic acid (TCA) to a final volume of the supernatant at −20 °C overnight. The precipitated proteins of conidia and germ tube were washed with cold acetone (−20 °C) 4 times to remove TCA [15]. The obtained proteins after washing were dissolved in protein rehydration buffer (8M urea, 25 mM DTT, 2% CHAPS), and protein concentration was measured by Bradford protein estimation assay. The extracted protein samples were stored at −80 °C until further use [21].

SDS-PAGE Analysis of Protein Samples

Protein samples extracted from conidia or germinating conidia were separated on 12% SDS-PAGE. Equal concentration of proteins from both the samples was precipitated using methanol and chloroform precipitation method. Pellets from both samples were reconstituted in Laemmli sample buffer (10% SDS, Tris–Cl (pH 6.8) and 20% glycine, 0.02% bromophenol blue), and beta-mercaptoethanol was added to sample buffer at the time of use. Protein samples and protein marker were heated at 95 °C for 5 min, followed by separation on 12% SDS-PAGE. Resolved proteins were stained overnight with Coomassie blue stain and destain thereafter. The gel was scanned on Bio-Rad G800 densitometer gel scanner (Bio-Rad). The gel image analysis and densitometry studies were carried out using Bio-Rad's Quantity One software, supplementary Fig. 1 [22].

Protein Digestion

The protein samples from conidia or germinating conidia of *A. terreus* were dissolved in 50 mM ammonium bicarbonate to obtain the required concentration of 1 mg/ml for LC–MS/MS-QTOF analysis using Acquity Waters UPLC system (Waters). The protein samples were analyzed at Sandor Life Sciences Pvt. Ltd, Hyderabad, India. For analysis, 100 µl of protein samples were precipitated with acetone followed by treatment with 10 mM DTT at 56 °C for 1 h. Further, samples were subjected to alkylation with 55 mM IDA at room temperature for 45 min in dark. Trypsin digestion was performed overnight at 37 °C for protein samples. Tryptic digest was eluted in 0.1% formic acid. An aliquot of protein samples was subjected to SDS-PAGE to analyze digestion, and remaining samples were subjected to BEHC18 column for peptide separation [15].

LC–MS/MS and Data Analysis

The separated peptide fraction was analyzed using Acquity Waters UPLC system (Waters). The peptides were separated on a BEHC18 column having size 2.1mm × 150mm × 1.7µm for 60-min run using buffers (buffer A: 0.1% formic acid and buffer B: 0.1% formic acid, ACN) at a flow rate of 0.2 ml/min. The separated peptides were ionized using ESI (Electrospray Ionization) at a temperature of 275 °C and spray volume of 2 kV. The mass spectrum was obtained in automatic MS/MS mode. The obtained spectra were analyzed using PLGS software (Waters Inc) [15]. The data were obtained with following parameters; enzyme trypsin with one missed cleavage having modification carbamidomethyl of cysteine and oxidation of methionine with peptide tolerance (ppm) 100 and fragment tolerance (ppm) 200. The data search was made against *A. terreus* database in UniProt.

Bioinformatics Analysis

Gene ontology (GO) annotation was done using Blast2GO algorithm, http://www.blast2go.de/. The GO Slimmer tool (http://amigo.geneontology.org) was used to obtain high level broader parent terms, GO molecular functions and cellular localization predictions also known as GO Slim terms [23].

Protein–Protein Interaction of Major Matched Pathways Proteins

Protein–protein interaction network is essential to reveal important function and to predict various cellular pathways involved in different morphological stages of *A. terreus*. Expressed proteins that are involved in cellular functions and pathways such as

signal transduction, carbohydrate metabolism and others were subjected to protein–protein interaction using STRING database version 10. The interaction of proteins was performed in different modes such as confidence view, interactive mode, evidence view and action view to construe the most suitable interactions among proteins and various function pathways [24].

Quantitative Real-Time PCR (qRT-PCR)

Total RNA from conidia and germinating conidia of *A. terreus* was extracted from two biological replicates using TRIzol reagent as per manufacturer's instruction (Invitrogen, USA). The quality and quantity of extracted RNA were assessed at A_{260} nm/A_{280} nm by using Nanodrop spectrophotometer (Thermo Scientific, USA). The integrity of extracted RNA was analyzed by electrophoresis using 1.2% agarose gel stained with ethidium bromide for the presence of intact ribosomal RNA bands (18S and 28S), visualized by UV transillumination at 302 nm. The cDNA synthesis was carried out from total RNA (1 µg) using verso-cDNA synthesis kit (Thermo scientific, USA) according to manufacturer's instructions. cDNA synthesis was performed at 42 °C for 30 min for reverse transcription followed by inactivation at 95 °C for 2 min. Primers were designed for selected gene for expression study using Primer-Blast tool at NCBI (Supplementary table-1) [25]. qRT-PCR was performed for selected genes (Bio-Rad machine CFX96, Bio-Rad, USA). The cDNA (1.5 µl in a total volume of 20 µl reaction) was used as template for qRT-PCR. All the reactions were performed in triplicate from two biological replicates using SYBER-Green master-mix (Thermo Scientific, USA) according to manufacturer's instructions. The thermal cycling conditions for qRT-PCR were: initial denaturation at 95 °C for 3 min, and 39 cycle of 95 °C for 10 s, 49 °C for 45 s, 72 °C for 30 s. A multi-curve was performed to check the specificity of primers. We observed insignificant difference in transcripts encoding for 40S ribosomal protein in conidia and germinating conidia, thus used as a reference gene to normalize the expression of selected genes (Supplementary table-1) [26]. We used "ΔΔCt" method to quantify the expression of selected genes in conidia and germinating conidia of *A. terreus* [13].

Results

Selection of Germination Time Point of *Aspergillus terreus* for Proteome Analysis

To analyze the developmental change in *A. terreus* conidia in DMEM medium, cells were microscopically evaluated at each hour interval. No significant morphological development was observed during first 9 h of inoculation. After that conidia expanded isotropically and swell at 10–11 h of incubation in DMEM medium (Fig. 2). Conidia continued to swell, and polarized growth was observed at 14 h. Approximately 90% conidia were observed to be germinated at 16 h of incubation as determined by microscopic count. After that the hyphae were developed and continued to elongate, branched and finally form mycelia network (Fig. 2).

Identification of Proteins During Germinating Conidia

To identify the expressed proteins during germination of *A. terreus* conidia (16 h), the extracted proteins were analyzed by LC–MS/MS. The obtained spectra were subjected to PLGS software for protein identification using data search in UniProt database. A total of 373 proteins specific to *A. terreus* were identified. Among them, 74 proteins were uncharacterized. The estimated sequence coverage of identified proteins ranged from 4.2 to 80.83%, and PLGS score ranged from 8.21 to 2993.38. Further the molecular weight of expressed proteins was ranged from 12,949 to 228,116 Da, and theoretical isoelectric point (pI) ranged from 4.1 to 11 (Supplementary file-1).

Further the gene ontology (GO) functions were assigned using BLAST2go software and UniProt database. Proteins were assigned a single GO term as biological processes, molecular functions and cellular component. Biological processes included protein biosynthesis (4.01%), protein transport (6.6%), transcription/regulation (5.0%), rRNA processing (7.6%), cell wall organization (3.01%), carbohydrate metabolism/catabolism (6.02%) and other biological processes as shown in Fig. 3. Further GO analysis revealed that the large fraction of protein expressed at germinating time point was dedicated to specific molecular functions including nucleic acid binding (14.7%), hydrolase activity (13.04%), oxidoreductase

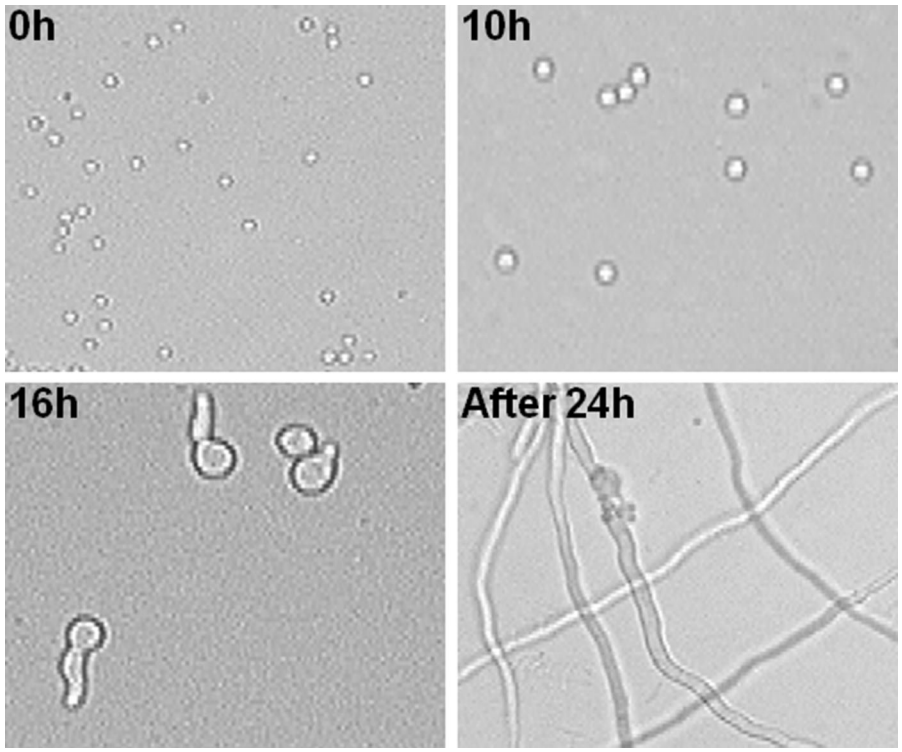

Fig. 2 Morphotypes of *Aspergillus terreus* during the growth from conidia to mycelium

(9.3%), transferase (7.02%), ATP binding (10.36%), and other significant fraction of expressed proteins are shown in Fig. 4. This analysis showed that most of the expressed proteins belonged to catalytic and binding activities. Cellular components of GO analysis showed that majority of expressed proteins were

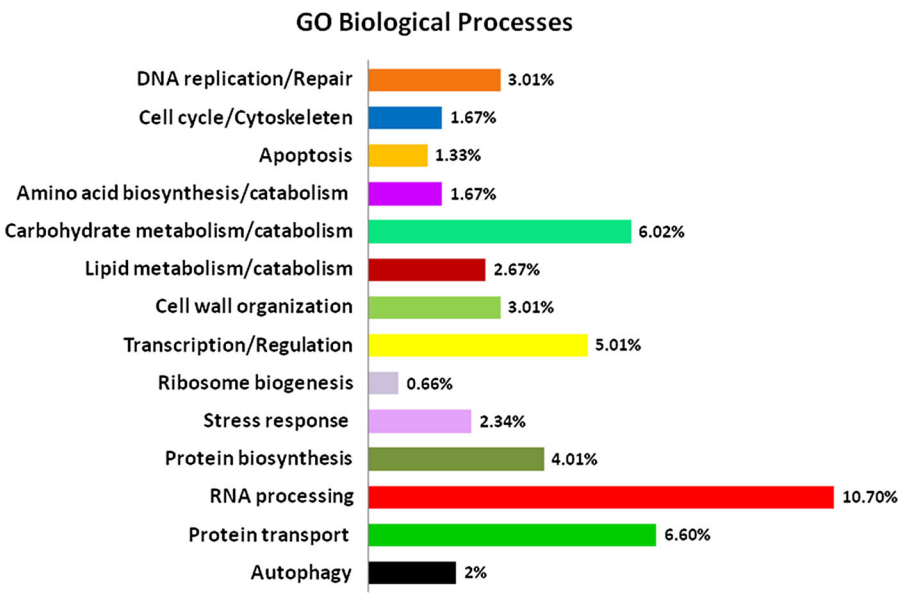

Fig. 3 Gene ontology (GO) enrichment (biological function) of expressed proteins in germinating conidia of *Aspergillus terreus*

belonged to cytosol (24.41%), nucleus (20.40%), extracellular (15.05%), mitochondrion (13.71%), endoplasmic reticulum (9.36%), and rest of the significant proteins of cellular components given in Fig. 5. Further, data from UniProt database has revealed proteins from different functional categories such as protein synthesis, amino acid metabolism, carbohydrate metabolism, energy production and conversion, lipid metabolism, posttranslation modifications, secondary metabolite biosynthesis and transport (Supplementary file-2).

Identification of Conidial Proteins of *Aspergillus terreus*

Conidial proteins of *A. terreus* were identified as discussed above, the extracted proteins were analyzed by LC–MS/MS and spectra were subjected to PLGS software for protein identification using data search in UniProt database against *A. terreus*. A total 42 proteins were identified; among them 17 proteins were uncharacterized (Supplementary File-3). No major difference in proteins were observed between germinating conidia and conidia of *A. terreus*. This may be due to the lesser number of proteins we could indentify in *A. terreus* conidia. The majority of proteins expressed in conidia were involved in secondary metabolite biosynthesis, protein synthesis and lipid metabolism etc (Supplementary file-4). Identification of uncharacterized proteins suggest that biological function of these proteins yet to be determined including their specific lineages in other fungal pathogens.

Proteins Associated with Germination of *Aspergillus terreus* Conidia

The analysis of expressed proteins of *A. terreus* germinating conidia permitted the recognition of a range of biochemical events and pathways associated with germination of *A. terreus* conidia.

Protein Biosynthesis and Amino Acid Metabolism

Proteins/enzymes that are involved in protein biosynthesis, amino acid metabolism, transcription, post-translation modification, protein transport and DNA replication were identified during germination of *A. terreus* conidia (Fig. 3). The protein synthesis requires a free pool of amino acids. We identified the proteins that involved in biosynthesis of arginine, methionine and polyaromatic amino acid biosynthesis such as ornithine carbamoyltransferase, pentafunctional AROM polypeptide, methylthioribulose-1-phosphate dehydrates, amino acid acetyltransferase and arginine biosynthesis bifunctional protein ArgJ during germination of *A. terreus* conidia. The identification of these proteins suggested that there may be specific

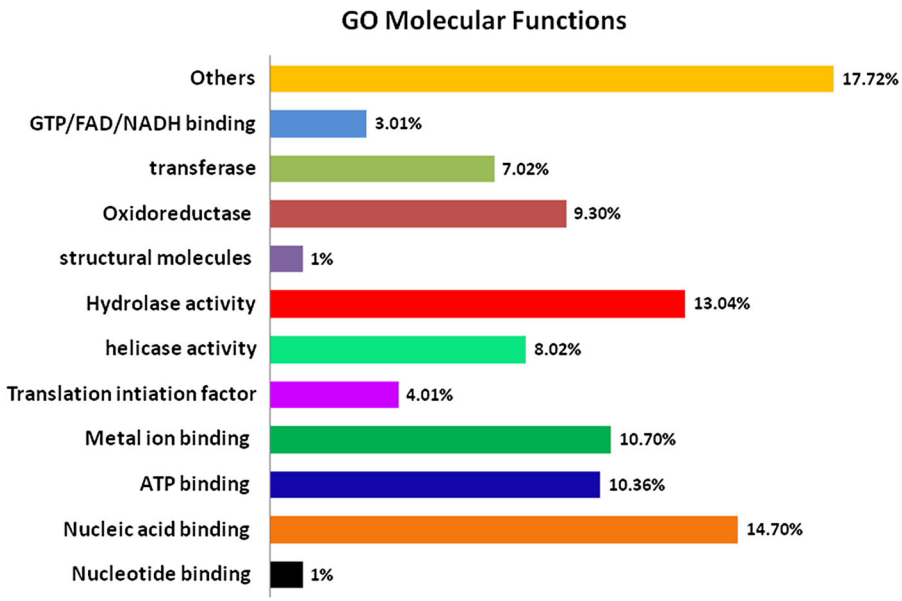

Fig. 4 Gene ontology (GO) enrichment (molecular function) of expressed proteins in germinating conidia of *Aspergillus terreus*

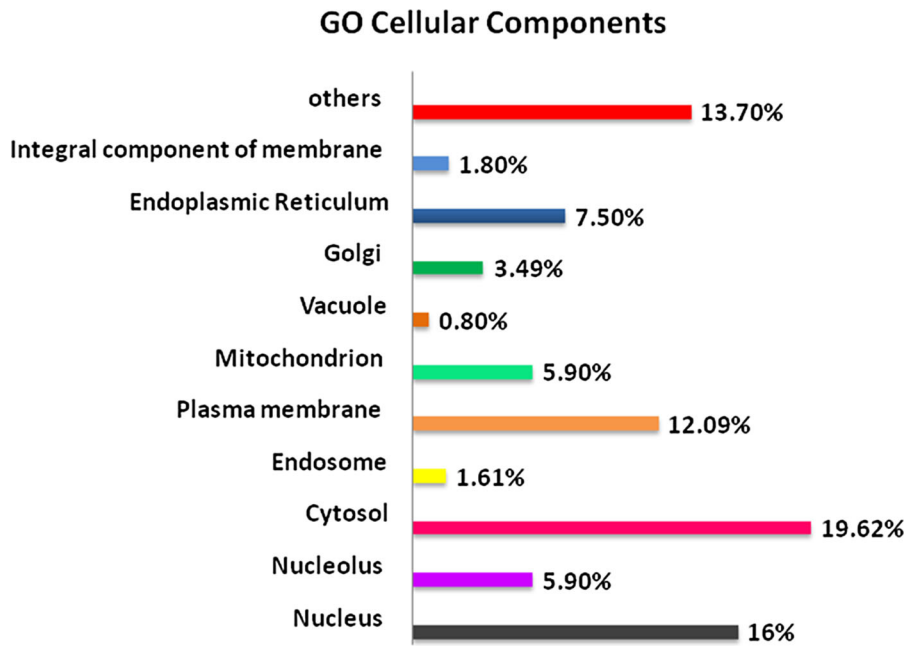

Fig. 5 Gene ontology (GO) enrichment (cellular function) of expressed proteins in germinating conidia of *Aspergillus terreus*

requirement of arginine during germination of *A. terreus* conidia. In addition, various translation factors required for protein synthesis were also identified during germination (Supplementary file-5).

Proteins Involved in Cell Wall Organization and Modification

Germination of fungal conidia leads to the remolding of cell wall-associated proteins. The cell wall of *Aspergillus* species mainly composed of β and α-glucan and chitin. Proteins that are involved in the degradation of cell wall components have been observed during germination of *A. terreus*. Enzymes such as glucan endo-1,3-beta-glucosidase, endopolygalacturonase, pectate lyase, alpha/beta-glucosidase and glucan endo-1,6-beta-glucosidase have been identified. Further the enzymes involved in carbohydrate catabolism were also identified and listed in Supplementary File-5 and 2. The orthologs of 1,3-β-glucan synthase (a component of FKS1), chitin synthase and endopolygalacturonase B were observed in *A. niger* and in *A. fumigatus*, respectively. Thus, the presence of these proteins revealed the organization and modification of cell wall of *A. terreus* during the germinating.

Identification of Probable Virulence Factors

Proteins involved in virulence were observed during germination of *A. terreus* conidia. Further there homologs were mapped to *A. fumigatus* using protein BLAST. These proteins were also reviewed in the literature to confirm their virulence in *A. fumigatus* knockout studies (Table 1). A total 5 virulent proteins have been identified as follow cgrA (rRNA-processing protein), creB (ubiquitin carboxyl-terminal hydrolase), pepP (Xaa-Pro aminopeptidase), myoA (myosin-1 OS), mep (metalloproteinase), hog1 (mitogen-activated protein kinase), and mpkC (mitogen-activated protein kinase).

Enzymes Involved in Secondary Metabolite Biosynthesis

Aspergillus terreus produces secondary metabolites ranging from mycotoxin (terretonin and geodin) to medically important biomolecules such as lovastatin and terrein. The enzymes involved in the biosynthesis of mycotoxins, (terretonin and geodin) have been observed. Enzymes from terretonin pathway such as methyltransferase (Trt5), FAD-binding monooxygenase (Trt3), terpene cyclase (Trt1), isomerase (Trt14), cytochrome P450 monooxygenase (Trt6),

Table 1 Identification of *Aspergillus terreus's* proteins reported as virulent factors in *Aspergillus fumigatus*

S. no	UniProt ID	Gene name	Description	PLGS score	Virulence factor reported in *A. fumigatus* studies
1	Q0C7E6	*cgrA*	rRNA-processing protein	514.8485	Bhabhra et al. [27]
2	Q0CEX5	*myoA*	Myosin-1	208.5809	Mcgoldrick et al. and Renshaw et al. [28, 30]
3	Q0CFJ0	*mep*	Extracellular metalloproteinase	191.1076	Hohl et al. [31]
4	Q0D0P5	*hog1*	Mitogen-activated protein kinase hog1	156.7995	Bruder et al. [29]
5	Q0CIC7	*mpkC*	Mitogen-activated protein kinase mpkC	143.4724	Bruder et al. [29]

Table 2 Expression of terretonin pathway enzymes in germinating conidia of *Aspergillus terreus*

UniProt ID	Gene name	Protein name	PLGS score
Q0C8A3	*trt5*	Methyltransferase trt5	249.0476
Q0C8A5	*trt3*	FAD-binding monooxygenase trt3	143.183
Q0C8A7	*trt1*	Terpene cyclase trt1	141.9876
Q0C8A2	*trt14*	Isomerase trt14	122.0085
Q0C8A1	*trt6*	Cytochrome P450 monooxygenase trt6	121.7647
Q0C898	*trt9*	Dehydrogenase trt9	102.1687
Q0C8A0	*trt7*	Dioxygenase trt7	71.9643

dehydrogenase (Trt9) and dioxygenase (Trt7) were identified from UniProt database. Other mycotoxin geodin pathway enzymes ACP thioesterase (GedB), anthrone oxygenase (GedH), methyltransferase (GedA), decarboxylase (GedI), atrochrysone carboxylic acid synthase (GedC), glutathione S-transferase (GedE), monooxygenase (GedF), oxidase (GedK), sulochrin halogenase (GedL) and dihydrogeodin oxidase (GedJ) have also been identified. Apart from mycotoxin pathway proteins, enzymes from lovastatin biosynthesis pathway and terrein biosynthesis pathway (an anticancer biomolecule) showed expression during germination of *A. terreus* conidia (Tables 2, 3 and Supplementary file-6).

Protein–Protein Interaction Network of Expressed Proteins

We performed the protein–protein interaction of expressed proteins because some of the proteins execute their biological functions by interacting with other proteins. To predict the function of those proteins, STRING database was used. We used GO (Biological process) of categorized proteins to generate an interactome map. Non-interacting proteins were eliminated from the interaction network (Supplementary figure-2). The interaction map showed that most of the interacting proteins were involved in protein transport, rRNA processing, protein biosynthesis, transcription/regulation, cell wall organization, carbohydrate metabolism and cell cycle. The list of interacting proteins is presented in supplementary file-7.

Quantitative Expression Analysis of Selected Genes of A. terreus in Germinating Conidia and Conidia

In order to quantify the expression of selected proteins at transcript level during germinating conidial stage, following genes *tif35* (eukaryotic translation initiation factor 3 G), *pcy* (pyruvate carboxylase), *gedJ* (dihydrogeodin oxidase) and *ATEG_03556* (terrelysin) were considered for expression study in conidia and in the germinating conidia. We observed significant upregulation of transcripts for terrelysin gene (885.28-fold), *pcy* (181.01-fold), *tif35* (148-fold) and *gedJ* (26.53-fold) in germinating conidia in comparison to conidia (Table 4).

Table 3 Expression of enzymes from geodin pathway during germination of *Aspergillus terreus* conidia

UniProt ID	Gene name	Description	PLGS score
Q0CCY4	*gedB*	Atrochrysone carboxyl ACP thioesterase	371.2632
P0DOB2	*gedH*	Anthrone oxygenase	102.6011
Q0CCY5	*gedA*	O-methyltransferase	255.0553
P0DOB3	*gedI*	Decarboxylase gedI	148.0289
Q0CCY3	*gedC*	Atrochrysone carboxylic acid synthase	133.797
Q0CCY0	*gedE*	Glutathione S-transferase-like protein	74.946
Q0CCX9	*gedF*	Monooxygenase	69.6025
Q0CCX5	*gedK*	Questin oxidase	36.546
Q0CCX4	*gedL*	Sulochrin halogenase	291.2784
Q0CCX6	*gedJ*	Dihydrogeodin oxidase	217.0705

Table 4 qRT-PCR analysis of transcripts of selected genes in germinating conidia in comparison with conidia of *A. terreus*

S. no	Gene name	Protein description	Fold difference in germinating conidia versus conidia (16 vs. 0 h)
1	*ATEG_03556*	Terrelysin	885.28
2	*tif35*	Eukaryotic translation initiation factor 3 G	148
3	*pcy*	Pyruvate carboxylase	181.01
4	*gedJ*	Dihydrogeodin oxidase	26.53

Discussion

Aspergillus terreus is an opportunistic pathogen worldwide. It appears as a frequent isolate in clinical samples in various parts of world such as M. D. Anderson Cancer Center in Houston, Texas, USA and the Medical University Hospital of Innsbruck, Austria [27, 28]. In a recent epidemiological study, the prevalence of *A. terreus* isolates (6.6%) has been observed among *Aspergillus* infection in the duration of 4 years at referral chest hospital, Delhi, India [6, 29]. Often *A. terreus* causes fatal disseminated infection in severe immunocompromised individuals [4]. Inhaled *A. terreus* conidia may persist in immune cell if not cleared, disseminate to secondary organs. Further, it is difficult to diagnose *Aspergillus* or fungal pathogens at an early stage due to lack of suitable diagnosis method or awareness [30, 31]. For the diagnosis of *Aspergillus* associated infection, cell wall-associated antigens are employed. Anigen based diagnosis is a better option as compared to antibody based detect because after infection antibodies may remain in patients for long time period or in immunocompromised individuals the antibody response is generally poor [32]. *Aspergillus terreus* diagnosis is important due to its resistance to Amphotericin B [33, 34]. Thus, proteins profiling during germination of *A. terreus* conidia not only provide the insight into the germination mechanism but may also lead to the identification of biomolecules for better diagnostic markers or vaccine candidates.

Aspergillus terreus employs unique strategy to evade human immune system. The prolonged persistence inside immune cells such as macrophage helps pathogen to escape from immune system or immune cell surveillance [4]. When the individual's immunity is compromised, conidia start to germinate and establish fatal invasive pulmonary or secondary organ infections [7]. Invasion of host tissues needs virulence factors, and among *Aspergillus* species, several virulence factors have been reported in *A. fumigatus.* During germination of *A. terreus* conidia, we have identified various virulence proteins such as mitogen-activated protein kinase (mpkC), mitogen-activated protein kinase (hog1), rRNA-processing protein (cgrA) and myosin-1 that are reported as virulence factor in *A. fumigatus* [35–39]. Further various proteins associated with cell wall organization have been identified, the protein involved in glucan synthesis and metabolism such as 1,3 beta-glucan

synthase component FKS1, probable glucan endo-1,3-beta-glucosidase btgC, probable glucan endo-1,6-beta-glucosidase B, mitogen-activated protein kinase hog1 and rhamno-galacturonate lyase B. Previously it has been observed that the *A. terreus* conidia and pre-swollen conidia display the β-1,3-glucan that assist in phagocytosis by alveolar macrophages in lungs and allow persistence of *A. terreus* conidia inside phagocytic cells [4]. Further, using qRT-PCR, it has been observed that the gene encoding for eukaryotic translation initiation factor (*tif35*) and pyruvate carboxylase (*pcy*) showed higher expression in germinating conidia in comparison with conidia (Table 4). These observations suggest that during transition of conidia to germinating conidia, the proteins/enzymes involved in protein synthesis and carbohydrate metabolism are required. Previously, Lamarre et al. [40] suggested that exit of conidia from dormancy is associated with a shift from a fermentative metabolism to a respiratory metabolism as well as a trend toward immediate protein synthesis. The protein interaction network of selected proteins of *A. terreus* using String database revealed that proteins involved in interaction were belonged to rRNA processing (*rrp3*, *dbp10* and *dbp3*), protein synthesis (*tif1, rps0* and *tif34*) and to protein transport (*sec13*). Interactome data indicated that rRNA processing, protein synthesis and protein transport facilitated conidia to exist from the dormancy. Furthermore, comparative analysis of expressed proteins of *A. terreus* germinating conidia with *A. fumigatus* and *A. flavus* suggested that during germination, protein/enzymes are required for the initiation of protein synthesis, carbohydrate metabolism and respiratory metabolism etc. (Supplementary table-2) [12, 15, 41].

Expression of enzymes involved in the biosynthesis of mycotoxin (terretonin and geodin) and other secondary metabolites (terrein, lovastatin and citreoviridin) were also observed during the germination of *A. terreus* conidia. Using qRT-PCR, we have observed the high expression of *gedJ* gene encodes enzyme dihydrogeodin oxidase of geodin biosynthetic pathway in germinating conidia as compared to conidia. These observations suggest that the pathway of geodin biosynthesis may be active at germination stage of *A. terreus* but role of geodin during the germinating stage needs investigation. Recently, Ozdemir et al. [42] suggested the use of mycotoxin as diagnostic marker for fungal infections, but the limitation of study was they did not tested the clinical samples. Further Lewis et al. [43] detected the gliotoxin in sera or other fluids in patients infected with *Aspergillus* species and suggested the screening of patient samples for mycotoxins as early diagnostic maker for invasive aspergillosis. Further studies are required on the prevalence of mycotoxins and their kinetics in sera or other fluids in patients infected with *Aspergillus* spp. or other toxin producing fungi. Further Koo et al. [44] explored other secondary metabolites such as volatile organic compounds for the diagnosis of *Aspergillus* species. Thus, the secondary metabolites have potential for evaluation or development of noninvasive diagnostic methods for fungal infections.

Previous studies on germinating conidia of *A. flavus* and *A. fumigatus* showed the expression of enzymes involved in the biosynthesis of aflatoxin, gliotoxin and other secondary metabolite, and may potentially participate in pathogenesis [15, 45–47]. Expression of enzymes of mycotoxin pathway in our study indicates the production of mycotoxin during germination of *A. terreus* conidia, which may further complicate the infection associated with *A. terreus*. Previously, Slesiona et al. [5] observed that the leukopenic mice infected with *A. terreus* conidia develop fatty liver degeneration after 48 h without any germination until 48 h but the germ line was observed at day 5 postinfection. These observations suggested that the secondary metabolites synthesized by *A. terreus* may be involved in fatty liver degeneration in the host. We also observed the expression of proteins involved in ergosterol biosynthesis, a target for antifungal drugs especially for azole group of drugs [48, 49].

Terrelysin, a hemolysin, was also identified during germination of *A. terreus* conidia that may contribute to virulence [50]. Further using qRT-PCR, it has been observed that gene encoding for terrelysin showed higher expression (885.28-fold) as compared to conidia. Data suggested that synthesis of terrelysin occurs during the germination of *A. terreus* conidia. Previously it has been reported that terrelysin expressed during early growth of *A. terreus* and reduced after mycelium formation and further it diffuses extracellularly into the medium maybe from apical region of hyphae or from germinating conidia [50]. Thus, identification of terrelysin protein suggests its role in invasion into the host tissues or organs. Terrelysin has been reported to lysis red blood cells and iron release,

which helps fungal pathogen for their growth and metabolic activity [51]. Further, as we have observed high expression of terrelysin, it may be explored as a diagnostic marker for the diagnosis of *A. terreus*-associated infections.

In conclusion, identification of proteins/biochemical pathways of *A. terreus* during germination has provided insight into the germination of *A. terreus* conidia. Further, we propose that expression of proteins/enzymes involved in biosynthesis of secondary metabolite could pave a way for development of metabolite-based diagnostic marker for *A. terreus*-associated infections [42, 52].

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Compliance with Ethical Standards

Conflict of interest The authors declare that no financial and competing interest exists.

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