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REVIEW ARTICLE

Genetics, Molecular, and Proteomics Advances in Filamentous Fungi

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Abstract Filamentous fungi play a dynamic role in health and the environment. In addition, their unique and complex hyphal structures are involved in their morphogenesis, integrity, synthesis, and degradation, according to environmental and physiological conditions and resource availability. However, in biotechnology, it has a great value in the production of enzymes, pharmaceuticals, and food ingredients. The beginning of nomenclature of overall fungi started in early 1990 after which the categorization, interior and exterior mechanism, function, molecular and genetics study took pace. This mini-review has emphasized some of the important aspects of filamentous fungi, their pattern of life cycle, history, and development of different strategic methods applied to exploit this unique organism. New trends and concepts that have been applied to overcome obstacle because of their basic structure related to genomics and systems biology has been presented. Furthermore, the future aspects and challenges that need to be deciphered to get a bigger and better picture of filamentous fungi have been discussed.

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Introduction

There are varieties of fungal species involved in most common opportunistic mycotic infection, mainly *Candida* spp. and *Cryptococcus neoformans* [102], while some serious infections occur due to *Aspergillus spp*. and other filamentous fungi [52, 67, 102]. These are becoming a threat to the medical and research field regarding infectious morbidity and mortality worldwide [36, 51, 101, 102]. The filamentous fungus mainly attacks immunocompromised host which worsens the health condition more seriously. Therefore, many studies on pathogenic fungus have been performed in recent years [55, 63, 86, 102]. Until 2000 AD, Amphotericin B was the standard therapy for mycotic infections caused by a number of hyaline filamentous fungi such as *Fusarium*, *Acremonium*, *Penicillium*, and *Scedosporium species*, *Zygomycetes*, and *Dematiaceous* filamentous fungi such as *Bipolaris*, *Alternaria*, and *Exophiala* species; however, the effect is only suboptimal. Hence, more research on active and potential therapeutics is needed. Because of the unique behavior and distinctive cellular organization, they have been creating extraordinary challenges in describing their form and function, which has been an essential factor for the breakout diseases [74]. The rigid hyphal network and the cytoplasm that can be moved within the hyphal network are the two peculiar characteristics that make filamentous fungi a unique group over all organisms. Moreover, the heterokaryotic nature of organisms [54] undergoing cell fusion and the growth mode resulting into hyphae with multinuclear cellular compartments represent another wicked setback in antifungal clinical setting [35, 91, 95]. In spite of these special features that are an obstacle from a therapeutic aspect, filamentous fungi are yet another important class of eukaryotic organisms of significant commercial relevance, especially

in the production of antibiotics, food additives, or recombinant proteins at the industrial scale [35, 72]. For example, the market value of cholesterol-lowering statins represents almost US$15 billion per year in the USA [72]. Fermentation of filamentous fungi for producing many enzymes, antibiotics, fermented food, pigments, medicine, and many useful compounds is most commonly used. Some filamentous fungi are potential producers of a wide range of lignocellulolytic enzymes, which has drawn great interest in many industries [43, 47]. Some of the fungi that are industrially important for the production of various enzymes are listed in Table 1. Based on previous publications, the study on filamentous fungi has been done in 1853 which advanced with the study of nomenclature in 1904. Until now, the number of studies on filamentous fungi has increased drastically with new advancement and technologies that have provided solutions to lots of health, environmental, industrial, and economical problems.

Table 1 List of some of industrially important fungi

Fungi	Enzymes	Fungi	Enzymes
Agaricus bisporus [28]	Cellulase, β-glucanosyltransferase, Glucose-6-phosphate dehydrogenase	*Macrophomina phaseolina* [120]	Endoglucanase, Lipolytic enzyme, Hemicellulolytic enzyme
Aspergillus fumigatus	[7, 8, 23, 27, 49, 80, 90, 126]	Endoglucanase, Cellulase, Xylanase, Mannosyltransferase	*Myrothecium* verrucaria [110]
Cellulolytic enzyme, Hydrolytic enzyme, oxidases			
Aspergillus niger [4, 9, 12, 26, 34]	Glucoamylase, Cellulase, tRNA-synthases, and protein transporters, Phytase	*Pellicularia filamentosa* [117]	Hemicellulase, Cellulase, Carbohydrate-degrading enzymes
Aspergillus terreus [58, 98]	Hydrolytic enzyme, Protease, Peroxidase	*Penicillium citrinum* [39]	Cellulase, Protease, Lignin peroxidases, Antibiotics, Organic acids
Aspergillus nidulans [68, 70, 108, 111]	Cellulase, Xylanase, Mannosidase, Transferase, Gluconic acid, Organic acids	*Penicillium funiculosum* [123]	Hydrolytic enzyme, Hemicellulase, Antibiotics
Fusarium solani [122]	Lipase, Glucoamylase, β-glucosidase, Lignocellulolytic enzyme	*Penicillium irensis* [31]	Polygalacturonase, Catalase, Antibiotics, Cellulose-degrading enzyme
Humicola insolens [16]	Cellulase, β-glucosidase, Cellobiohydrolase, Galactosidase	*Penicillium janthinellum* [114]	Cellulase, β-glucosidase, Carboxypeptidase
Trichoderma koningii [53]	β-glucosidase, Cellulase, Lignocellulose	*Penicillium variabile* [98]	Cellulase, Antibiotics, Rugulovasine
Trichoderma lignorum [11]	Cellulase, Lipase, Hydrolytic enzyme	*Pestalotiopsis westerdijkii* [87]	Exocellulase, Glucosidase, Glucoamylase
Trichoderma longibrachiatum [105]	Cellulase, Xylanase, Pectolyase	*Polyporus adustus* [24]	Cellobiose oxidase, Cellulase, Quinone oxidoreductase
Trichoderma reesei [1, 3, 6, 38, 60]	Cellulase, Xylanase, Arabinofructofuranoside, Hemicellulases, Lignin-degrading enzymes, Proteases, Protein-translocating transporter, Mannosidase	*Polyporus tulipiferae* [66]	Endocellulase, Xylanase, Chitosanase
Trichoderma cutaneum [57]	Cellulase, Xylan-degrading enzyme	*Poria spp.* [115]	Endoglucanase, galactosidase, Lignin-degrading enzyme
Trichoderma atroviride [50]	Cellulase, Endoglucanase, Xylanase, β-glucosidase	*Sporotrichum dimorphosporum* [30]	Xylanase, Mannanase
Sporotrichum thermophile [32]	Cellulase, Hydrolytic enzymes	*Sporotrichum pulverulentum* [40]	β-glucanases, CMCase, Glucohydrolase
Talaromyces emersonii [87]	Glucosidases, Polysaccharide degrading enzyme	*Sporotrichum pruinosum* [104]	Cellulolytic enzyme, Bleaching enzyme

Life Cycle and Microscopic Observation of Filamentous Fungi

The complete life cycle of filamentous fungi lasts 2–3 laboratory weeks including both sexual reproduction (fusion of two haploid nuclei; karyogamy), followed by meiotic division of the diploid nucleus, and asexual reproduction (division of nuclei by mitosis) [25]. On the other hand, karyogamy may be followed immediately by the combination of two hyphal protoplasts (plasmogamy), or it may be separated in time [18, 25]. In contrast, identical haploid cells can be obtained by the asexual cycle, which can be used for mutagenesis and DNA-mediated transformation [25]. The schematic representation of the life cycle of filamentous fungi is presented in Fig. 1. Mainly, hyphal growth initiates by extension of hyphae at the tips followed by polarization [127]. The polarized growth can be partially identified by directional movement and vesicle accumulation carrying cell wall precursors and cell wall synthetases [25, 79, 127]. Ultrastructure study revealed that hyphal morphogenesis is a complicated organization of tip-growth-related organelles and cytoskeletal elements as well as chitin microfibrils at the apical dome of the hyphae [25, 63]. The chitosomes which control the activity of membrane-bound chitin synthetase may arise from Golgi-like bodies or by a process of self-assembly of subunits freely within the cytoplasm or within larger vesicular bodies. [25]. Many studies performed on microscopic observation of filamentous fungi including fluorescence microscopy, electron microscopy, or even confocal microscopy have successfully obtained the clear picture of the life cycle and reproduction process of filamentous fungi. Similarly, tagging of chimeric green fluorescent proteins (GFPs) to the target protein sequence of AfMp1, AfGel1, and AfEcm33, respectively, has also been proved to be an important method for identifying localizations of certain proteins based on fluorescence tag [97]. Phosphomannose isomerase (Pmi1) [41], GDP-mannose pyrophosphorylase [62], and O-mannosyltransferase 1 (AfPmt1) [129] in *A. fumigatus* are found to be crucial for cell wall integrity and conidium morphology, while GPI-anchor is essentially required for morphogenesis and virulence [78]. In addition, glucosidase I (AfCwh41) [127] and α-mannosidase (AfMsdC) [79] are required for cell wall synthesis, conidiation, septation, and polarity in *A. fumigatus*.

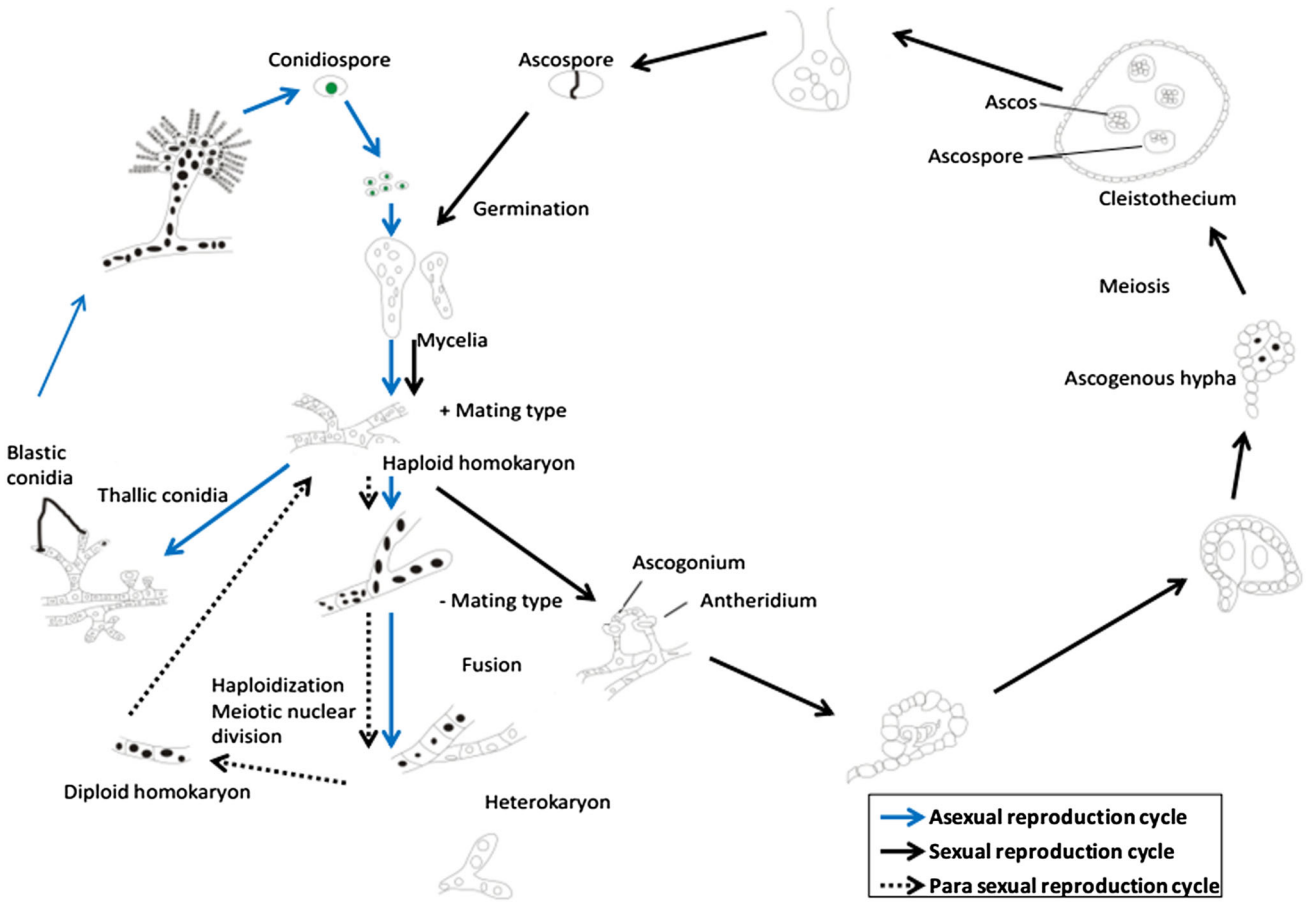

Fig. 1 Life cycle of filamentous fungi. *Blue arrow* indicates asexual reproduction, *black arrow* indicates sexual reproduction and *dotted black arrow* indicates para sexual reproduction cycle

Sexual reproduction in fungi typically involves the fusion of two haploid nuclei (karyogamy), followed by meiotic division of the resulting diploid nucleus. In some cases, sexual spores are produced only by fusion of two nuclei of different mating types, which necessitates prior conjugation of different thalli. This condition of sexual reproduction is known as heterothallism, and the nuclear fusion is referred to as heterokaryosis [25, 29]. Normally, plasmogamy (union of two hyphal protoplasts which brings the nuclei close together in the same cell) is followed almost immediately by karyogamy [25]. In certain members of the Basidiomycotina, however, these two processes are separated in time and space, with plasmogamy resulting in a pair of nuclei (dikaryon) contained within a single cell. The development of a dikaryotic mycelium results from simultaneous division of the two closely associated nuclei and separation of the sister nuclei into two daughter cells [25]. An alternative mechanism of sexual reproduction in the fungi is homothallism, in which a nucleus within the same thallus can fuse with another nucleus of that thallus (i.e., homokaryosis) [25, 29]. An understanding of these nuclear cycles is fundamental to the investigation of fungal genetics. Despite the absence of meiosis during the life cycle of these imperfect fungi, recombination of hereditary properties and genetic variation still occur by a mechanism called parasexuality [25]. The major events of this process include the production of diploid nuclei in a heterokaryotic, haploid mycelium that results from plasmogamy and karyogamy, multiplication of the diploid along with haploid nuclei in the heterokaryotic mycelium, sorting out of a diploid homokaryon, segregation and recombination by mitotic crossing over, and haploidization of the diploid nuclei. Some fungi that reproduce sexually also exhibit parasexuality, which could also provide genetic remuneration of meiosis that is achieved through mitotic means [25, 103].

Genetic Engineering and Molecular Approach

According to DNA analysis, the history behind the successful study of this unique group of organisms started since fungi diverged from other life around 1500 million years ago [20]. The typical features of fungi corresponding to earliest fossils possessing date belong to the Proterozoic eon [some 1430 million years ago (Ma)] where the multicellular benthic organisms were found to posses filamentous structures with septa and were capable of anastomosis [21]. In earlier 1930s, the genetic study of any organism was mainly based on mutation. The first heterologous gene of filamentous fungus *N. crassa pyr4* gene was isolated by Buxton and Radford in 1983 by complementation of *E. coli pyr* (*F*) mutant [22]. Earlier studies already revealed that in higher eukaryotes genetic studies through mutation can be accompanied by light microscopy for the determination of the karyotype. However, small chromosome size in fungi appeared to be problematic for microscopic studies of their karyotype. Thus, the mutation using different mutagens was the ultimate method for the genetic study. But the major problems with this method were the induction of chromosome aberrations with single gene mutations by mutagens [118] and the reduction of interchromosomal recombination and falsely indicated mitotic linkage [65]. Further, the genetic map could only give accurate genesis only if the species is genetically well understood which was lacking during that era. Teow et al. somehow solved those problems alternately using spontaneous mutations in the ascomycete *A. nidulans* as a model organism [119]. Previous studies have identified sequence and annotation of 18 different species of filamentous fungi such as *A. clavatus* [72, 124], *A. flavus* [93, 99], *A. fumigatus* [45, 93], *A. nidulans* [45], *A. niger* [100], *A. terreus* [124], *F. verticillioides* [19], *N. crassa* [17, 45], and *P. chrysosporium* [85]. Moreover, ten genome sequences of the most important industrial and medical Aspergilli are publically accessible, making this genus one of the best to be studied by comparative genome analysis [72]. The trends of genomics studies done on filamentous fungi in the last 20 years are depicted in Fig. 2a. Nevertheless, the great effort of researchers brought a fascinating period of new discoveries and breakthroughs in many new genetic tools and techniques in the past decades such as efficient genetic transformation systems, expression systems for high-level and controlled protein production, high-throughput gene targeting tools, and even live imaging techniques for cell structures [89].

PEG-mediated transformation system was first established in yeast (*Saccharomyces cerevisiae*) in 1978 [69], which was later followed by transformation of filamentous fungi in 1989 for the first time [42]. Although asexual spores such as conidia or sporangia are most favorable for filamentous fungi, sometimes the use of mycelia and multinuclear state of conidia make the transformation system in filamentous fungi less efficient than in yeast [10]. Later in late 90s, Chakraborti and Ruiz-Diez studied electroporation for the transformation using protoplast, conidia, or young germlings [33, 106]. A great progress in microbial metabolomics has been achieved in the last 37 years. However, it is clear that there appears to be no universal methodology in microbial metabolomics for instantaneous quenching of microbial metabolic activity, extraction of all low-molecular weight metabolites, and analysis of these metabolites of interest. The use of genomics, transcriptomics, proteomics, and metabolomics toward an improved and novel understanding of the biochemical processes has been proved to be important for

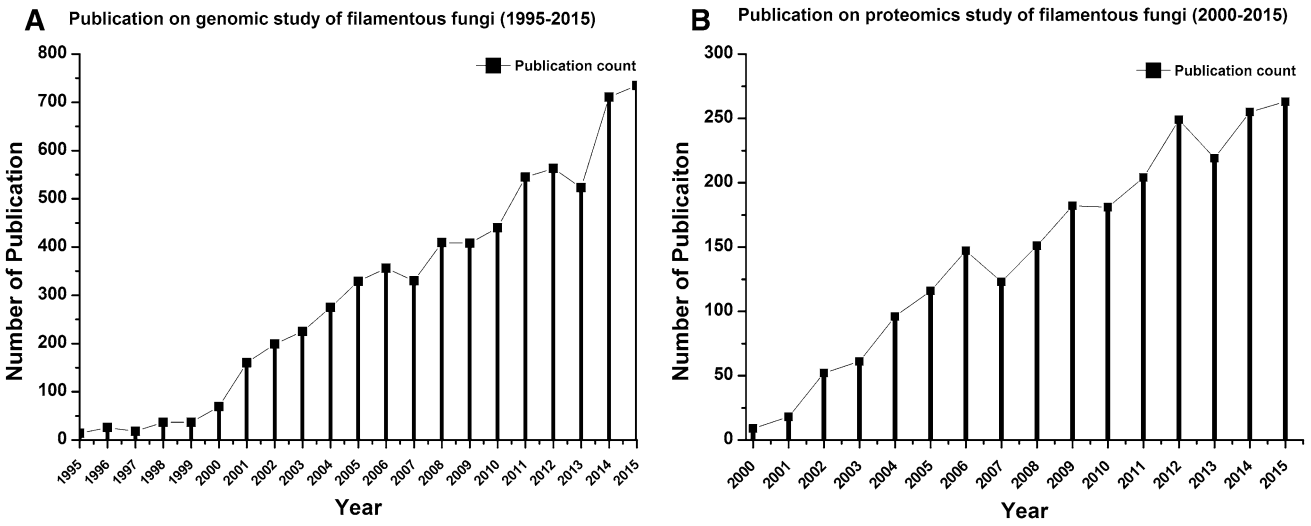

Fig. 2 Trends in genomics and proteomics study done on filamentous fungi

understanding the mechanism and massive overproduction of secreted proteins [75].

A number of genetic and genomic tools have been developed to obtain modified and improved strains to enhance the production of specific enzymes from fungi. Zou et al. [130] showed the direct engineering of the *cbh1* promoter of *T. reesei* by replacing the three binding sites of the carbon catabolite repressor CREI with binding sites of different transcription activators to improve the strength of promoter. The heterologous expression of thermostable endoglucanase E1 from *A. cellulolyticus* in *T. reesei* showed that the fusion proteins greatly improved the quality of carbohydrate metabolic enzyme with increased enzyme activity and better thermostability to release sugars from complex biomass. Similarly, molecular techniques such as cloning or denaturing gradient gel electrophoresis (DGGE) have provided better knowledge about microbial community structure [48]. A limited knowledge on microbial enzymes because of the narrow traditional microbiological and biochemical methods to characterize the enzymes of complex sugar degradation has been solved by much advanced genetic and molecular technology.

Yet another alternative strategy for gene targeting and in particular gene deletion has been in use in recent years. This strategy was also found to be more effective as compared with the tedious gene knockout strategies [116]. The RNA-based methods that silence gene expression post-transcriptionally are especially helpful either when gene targeting approach could not be attained, isogenes might compensate for the knockout, or when multiple copies of a gene of interest are present in the genome [88]. Many successful stories on gene silencing using artificial antisense constructs have been reported on filamentous fungi [15, 73, 92, 128]. Similarly, the effects of silencing of the transcriptional regulator toward the total expression of xylanases in *A. niger* was studied by measuring the relative expression levels of two highly transcribed target genes encoding D-xylose reductase and endo-β-1,4-xylanase B [82]. This method described homologous recombination between a general destination vector and a specific entry clone to generate the corresponding dsRNA expression [82]. Likewise, RT-PCR analysis revealed the inducible expression of an RNAi construct for efficient gene expression in *A. fumigatus* [71, 96]. In *T. koningii* YC01, a cellulase-hyperproducing strain with genetic similarity to *T. reesei*, the improved expression and production of cellulase and xylanase was studied by constructing ACEI through RNAi [121]. Similarly, CRISPR–Cas9 is another powerful and most recent approach for genome editing in a variety of organisms including filamentous fungi. CRISPR/Cas9 system has been studied in *Trichoderma reesei* by specific codon optimization and in vitro RNA transcription through inducible Cas9 expression. This system can generate site-specific mutations, even using short homology arms in target genes through efficient homologous recombination. This tool also provided an applicable and promising approach to target multiple genes simultaneously [77, 81, 94].

Proteomics Study on Filamentous Fungi

Several fungi possess exceptional capacity for protein production which provides one of the important aspects for identifying the protein function. The ideal targets for protein could be more easily exposed by proteomics techniques rather than conventional methods. The proteomic study has opened a method for screening the secreted

protein through their peptide sequence. The published data reveal that the progress particularly in proteomics and its techniques has been high only after 2000 (Fig. 2b). In the last few years, the secreted lignocellulolytic enzymes of individual strains and their co-cultures were analyzed by high-throughput isobaric tag for relative and absolute quantification (iTRAQ) coupled with liquid chromatography tandem mass spectrometry (LC–MS/MS) [5, 112]. Using 2D gel electrophoresis, Sato et al. [107] identified 18 proteins with the expression patterns of cellulolytic proteins from *P. chrysosporium* in cellulose-grown cultures and wood-grown cultures. It was reported that there are several disadvantages such as reagent cross-reactivity and detection sensitivity with colorimetric estimation and identification of enzymes in a complex secretome. Gene expression technologies such as transcriptome profiling are somehow flawless and advantageous but also include limitations such as RNA stability, choice of primer set, and a high number of false-positive and false-negative findings. However, proteomic technology is much more advanced, sensitive, and suitable to identify various sets of proteins from intricate biological samples [83, 84]. In recent years, an iTRAQ-based quantitative proteomics is a method of choice for proteomics analysis in order to understand cytosolic and membrane proteins, cellular process mechanisms, their regulations and protein–protein interactions, and possible metabolic pathway for enhanced cellulose hydrolysis potential of bacterial and fungal secretomes [5]. The proteomics analysis of *P. chrysosporium*, *T. fusca*, *A. nidulans*, *A. fumigatus*, *A. niger*, and *T. reesei* and other bacterial and fungal enzymes has provided a useful means to improve the understanding of their unique enzyme system and evaluate their use in industries for lignocellulosic bioenergy [3–5, 59, 80, 83].

The genome analysis of *T. fusca* exposed 45 hydrolytic enzymes, 28 putative glycoside hydrolases, and other enzymes optimally active at 55 °C [5, 61, 76]. It is assumed that membrane proteins could exhibit a major function in many biological processes, secretory protein export, nutrient transport, and signal transduction to provide special cellular strategies to survive and secrete thermostable hemi/cellulolytic enzymes. Cellulase genes present in *T. reesei* and *A. niger* have been identified from secreted protein by 2DGE and LC–MS [37, 64]. The protein secretion profile of *A. niger* identified 102 unique proteins including many hydrolyzing enzymes, such as cellulases, hemicellulases, hydrolases, proteases, peroxidases, and protein-translocating transporter proteins [4]. Moreover, most of the hydrolases have potential application in lignocellulosic biomass hydrolysis for biofuel production. For example, some enzymes such as endoglucanase, glucan 1,4-α-glucosidase, β-mannosidase, glycosyl hydrolase, proteases, and other enzymes like cytochrome C oxidase and glucose oxidase were highly expressed in *A. niger,* which have a major role in cellulolysis. In addition, specific enzyme production can be stimulated by controlling pH of the culture medium [2, 4, 9, 26]. However, the role of PTMs is not very clear although Adav *et al.* [59] showed deamidation in the secretome of *A. fumigatus*.

In LC-based platform, the proteins are first digested into peptides, followed by separation with strong cation exchange and then with reversed phase chromatography [125]. Numerous labeling methods depending on heavy isotopes such as ^{2}H, ^{13}C, ^{15}N, and ^{18}O have been developed and allow relative quantitation using MS. There are various quantitative methods that can be used in LC-based approach including stable isotope labeling by amino acids in cell culture (SILAC), cleavable isotope-coded affinity tag (cICAT) labeling, and isobaric tags for relative and absolute quantitation (iTRAQ). In iTRAQ approach, a corresponding balance group and an amine-reactive group are used to label primary amino group in peptides using either 4-plex or 8-plex isobaric tags consisting of a reporter mass of 114-117 (4-plex) or 113-121 (excluding 120, 8-plex). The labeled peptides are pooled, fractionated, and subjected to mass spectrometry whereby MS/MS fragmentation releases the reporter ions which would reflect the relative abundance of the proteins [56, 125]. In iTRAQ labeling for relative and absolute quantitation, the iTRAQ reagents react with primary amines of amino termini or lysine residues and label most peptides and proteins present in cells. iTRAQ reporter ions are released upon collision-induced dissociation (CID) and their relative intensities are used for protein quantitation. In contrast to ICAT and SILAC (where only two or three samples can be compared), iTRAQ permits labeling and quantitation of four or eight samples. Further, by mixing multiple samples in a single run, the instrument time for analyses can be reduced, and the results are not affected by variations between different LC/MS runs. Among these three approaches, iTRAQ has a higher sensitivity [46]; however, the three methods covered cell lysates' protein profiles which have little overlaps, suggesting that these methods may complement each other [14, 125].

Recent Advancement and Future Prospect

It is clear that the formation of heterokaryons in filamentous fungi leads to the formation of hyphae with multinuclear cellular compartment and webs of mycelia that limit the research of their cell cycle pattern [54, 91, 95]. Moreover, because of this nature the genetic and molecular techniques usually involve more laborious, tedious, and/or time-consuming procedures with greater risk of failure

[109]. However, in recent years Delgado-Ramos et al. studied flow cytometry procedure in yeast and filamentous fungi by microencapsulation of single spore in which growth was monitored by light or fluorescent microscopy and complex object parametric analyzer and sorter large-particle flow cytometry [35]. Similarly, Beneyton et al. developed a high-throughput screening method of filamentous fungi using nanoliter-range droplet-based microfluidics [13]. These represent a great achievement in the production of industrially important strains with low cost, space, and time which could bring enormous benefits for improving the genetic and molecular viability in filamentous fungi [35]. Furthermore, live cell imaging of hyphal fusion (anastomosis) process in growing colonies of *N. crassa* was also successfully observed by staining with the membrane-selective dyes FM1-43 and FM4-64 [13, 55]. In addition, the apoptosis occurring in these fungi was performed by fluorescent labeling of the nuclei and analyzed by SCAN©, a System for Counting and Analysis of Nuclei [113]. These researches provide a great value as nuclei are divided between compartments in filamentous fungi containing a number of nuclei which further migrate between the compartments [35, 113]. A versatile technique in which RNA-guided mutagenesis by transforming a target fungus with a single plasmid has also been successfully performed in yeast, *Aspergillus sp.* and *Trichoderma sp*. by using CRISPR-Cas9 vectors furnished with fungal markers permitting the selection in a broad range of fungi. This method definitely helps in improving the efficiency and stability of recombination and transformation and also improves the gene targeting or gene replacement in filamentous fungi [44, 77, 94]. However, many new and advanced methods have been applied in the study of filamentous fungi; still more studies are required for fully understanding the further mechanism of their cell cycle. The mechanism of their polarized growth and the relation of their growth with their metabolism such as carbohydrate metabolism or change in glycosylation still need lots of efforts to be fully understood. For this, better and efficient methods should be developed because the filamentous and multinucleated nature of filamentous fungi always creates a mystery and complexity in gaining better understanding.

Conclusion

Filamentous fungi can be categorized not only as a pathogenic microorganism but also as a microbial cell factory or the source of enzymes, chemicals, and pharmaceuticals. With the great effort of researchers and scientists, genetic engineering and molecular approaches have been devised to understand their unique features and functions. Although many techniques and strategies including DNA–RNA and genomics-based approach, protein and enzymes approach using various biotechnological aspects have been successfully used to exploit the basis of filamentous fungi, still more efficient, stable, valid, and productive tools are essentially required to solve the obstacles in having a better insight into many industrially and commercially important filamentous fungi so as to improve their productivities. This will in future unlock the door for further improving the strain to obtain the product of our interest. Furthermore, in future the fungal genomics, transcriptomics, and proteomics could even provide a network for profiling their importance in relation to other organisms including human and other prokaryotes and eukaryotes.

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