IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 11, Issue 4 Series 1 (Jul. – Aug. 2025), PP 06-12
www.iosrjournals.org

"Challenges in Fungal and Mushroom Genome Extraction: Technical, Biological, and Methodological Perspectives"

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ABSTRACT:
Fungi, including mushrooms, constitute a taxonomically rich and functionally diverse kingdom with significant ecological, industrial, and pharmaceutical relevance. Genomic studies in fungi have expanded rapidly, yet the extraction of high-quality genomic DNA remains a critical and technically challenging step. These difficulties stem from the rigid and multilayered composition of fungal cell walls, the abundance of inhibitory substances such as polysaccharides and secondary metabolites, and species-specific ecological adaptations. Such factors can compromise DNA yield, purity, and the efficiency of downstream molecular applications including taxonomy, phylogenetics, genome sequencing, and biotechnological research. This review critically examines the biological, technical, and methodological obstacles encountered during fungal and mushroom DNA isolation. It highlights recent advancements in mechanical disruption strategies, chemical lysis formulations, and commercially available extraction kits designed to enhance DNA recovery and integrity. Additionally, the paper discusses the need for developing species-adapted and field-compatible protocols, particularly for diverse mycological specimens. Emphasis is placed on the standardization of extraction methodologies to ensure reproducibility, data accuracy, and scalability in fungal genomic research. Finally, emerging trends such as automation and protocol customization are explored as promising solutions to address the current limitations in fungal DNA extraction techniques.

KEY WORDS:
Fungal, Genomics, Mushroom, DNA, Cell, Metabolites, Molecular, Extraction, Mycological Biodiversity, PCR, Inhibitors, Biotechnological ETC.

Date of Submission: 02-07-2025 Date of Acceptance: 12-07-2025

LITERATURE OF PAPER:
• **MINOU NOWROUSIAN & JASON E. STAJICH [FUNGAL GENOMICS (3RD ED., 2024)]:** Delves into high-throughput genetics, RNA editing, codon-mediated regulation, and epigenetics in early diverging fungi.

I. INTRODUCTION:

Fungi, including mushrooms, constitute a highly diverse kingdom of organisms that play vital roles in ecosystems, industry, and pharmaceuticals. One of their most significant contributions is the production of secondary metabolites (SMs)—a wide array of low-molecular-weight organic compounds synthesized from basic precursors. Although these compounds are not essential for the basic growth or reproduction of fungi, they often provide a competitive advantage by enhancing survival and adaptability. Despite the discovery of numerous fungal SMs, a vast number remain unidentified due to the immense diversity of fungal species and the technical challenges involved in SM detection. To date, only about 120,000 fungal species have been formally described, which represents less than 8% of the estimated global fungal diversity.[1] Fungi and mushrooms synthesize a broad spectrum of SMs, ranging from those beneficial for pharmaceutical, agricultural, and cosmetic applications to those detrimental to human and environmental health. For instance, plant-pathogenic fungi produce phytotoxins that can damage crops, while mycotoxins pose serious risks to food safety, livestock, and human health. Conversely, fungi are also the source of important therapeutic agents such as lovastatin and taxol, which are produced through unique biosynthetic pathways and used to treat conditions like hypercholesterolemia and cancer. These SMs are generally categorized into major classes such as polyketides, terpenoids, and non-ribosomal peptides, depending on their biosynthetic origins.[3] The genes responsible for the synthesis of these metabolites are often grouped together in the genome as biosynthetic gene clusters (BGCs). These clusters typically include core biosynthetic genes, regulatory elements, transport-related genes,

and self-resistance factors. Advances in sequencing technologies have greatly expanded the availability of annotated fungal genomes, accelerating BGC discovery through bioinformatic tools like antiSMASH, MIBiG, and BiG-SCAPE. Studies analyzing large genomic datasets have revealed significant variability in both the number and size of BGCs among fungal species, ranging from small clusters with a couple of genes to extensive ones spanning up to 100 kb.[2] Despite the progress, understanding the relationship between BGCs and their associated SMs remains limited. Many BGCs remain transcriptionally silent under standard laboratory conditions, and others have yet to be investigated. Strategies to activate these silent clusters are essential for uncovering new metabolites. Environmental cues, global transcriptional regulators, and cluster-specific transcription factors all influence BGC expression. Key regulatory complexes such as the velvet complex, BrlA, LaeA, and McrA have been identified as critical to the activation of multiple BGCs. Additionally, genetic modifications such as promoter replacement or transcription factor overexpression have proven effective in activating silent clusters. In *Aspergillus terreus*, for example, manipulating specific transcription factors has led to the production of novel compounds like naphthoquinones and azaphilones.[7] Epigenetic regulation also plays an important role in BGC expression, involving mechanisms such as DNA methylation, histone modification, small RNA expression, and chromatin remodeling. Reprogramming the fungal epigenome represents a promising approach to boost secondary metabolite production. To further explore active BGCs, researchers often generate gene knockout strains and analyze resulting metabolite profiles. Understanding the regulatory frameworks that control BGC activity offers a foundation for engineering fungi for enhanced SM biosynthesis.[5] This review provides a comprehensive overview of fungal secondary metabolism, beginning with an introduction to the roles and biosynthesis of SMs and their associated gene clusters. It also highlights traditional genetic engineering techniques used in fungi and focuses in depth on recent advances in CRISPR/Cas-based genome editing as a powerful tool for improving SM production. Furthermore, the strengths and limitations of different CRISPR/Cas systems are discussed, and future directions are proposed to enhance their application in fungal biotechnology, particularly for mushrooms and other lesser-studied taxa.

II. BIOLOGICAL CHALLENGES IN FUNGAL GENOME EXTRACTION:

2.1.COMPLEX CELL WALL COMPOSITION:

Fungal cell walls, including those of mushrooms, are composed of a structurally complex and chemically diverse matrix that presents a significant barrier to efficient DNA extraction. The primary components include chitin, β-glucans, mannoproteins, and sometimes melanin, which together form a rigid and resilient outer layer. Chitin, a long-chain polymer of N-acetylglucosamine, provides mechanical strength, while β-glucans and glycoproteins contribute to structural integrity and cross-linking within the wall. In many species, additional substances such as melanins or hydrophobins further reinforce the wall and protect against environmental stress. This intricate architecture not only varies between fungal taxa but can also change depending on developmental stages or environmental conditions.[8] Such variability complicates the lysis process, as conventional chemical or enzymatic methods often fail to sufficiently break down the wall, resulting in low DNA recovery or degraded samples. As a result, effective DNA extraction from fungi often requires a combination of mechanical disruption (e.g., bead beating or cryogenic grinding) and tailored enzymatic treatments to penetrate and dismantle the tough cell wall structure

2.2.SECONDARY METABOLITES AND POLYSACCHARIDE CONTAMINANTS:

One of the major obstacles in fungal and mushroom genomic DNA extraction is the presence of secondary metabolites and polysaccharides, which often act as contaminants that interfere with the quality and usability of the extracted DNA. Secondary metabolites such as phenolic compounds, pigments, alkaloids, and terpenoids are produced by fungi for defense, competition, and survival. While biologically important, these compounds can co-extract with DNA and bind to it, causing shearing or degradation. Similarly, polysaccharides, which are abundant in the fungal extracellular matrix and cell wall, can co-precipitate with nucleic acids during extraction, leading to viscous DNA solutions that are difficult to pipette and quantify accurately. These contaminants can inhibit enzymatic reactions such as PCR, restriction digestion, and sequencing, reducing the efficiency and reliability of downstream molecular applications. The problem is particularly pronounced in mushrooms due to their rich biochemical profiles and high water content, which can exacerbate co-extraction. Therefore, effective extraction protocols often include purification steps such as CTAB (cetyltrimethylammonium bromide) treatment, ethanol precipitation, or the use of binding columns to selectively isolate pure DNA and eliminate interfering compounds.

2.3.LOW DNA YIELD DUE TO DIFFICULTY IN EFFECTIVE CELL LYSIS:

One of the primary biological challenges in extracting genomic DNA from fungi and mushrooms is achieving effective cell lysis, which directly impacts DNA yield. Fungal cells possess a highly resilient and complex cell wall composed of chitin, β-glucans, mannoproteins, and other polysaccharides, which create a

rigid barrier protecting the intracellular contents. This multilayered structure is much tougher than that of bacterial or plant cells and significantly hinders the penetration of lysis buffers and enzymes commonly used in DNA extraction protocols. Because of this robust barrier, standard chemical lysis methods often fall short in breaking down the fungal cell wall completely. Incomplete lysis leads to inefficient release of DNA, resulting in low yields. Moreover, certain fungal species or developmental stages may produce thicker or more cross-linked walls, further complicating the lysis process. For instance, mushrooms in their mature fruiting body stage have densely packed cells and reinforced walls, making mechanical disruption more critical but also more difficult to optimize. Mechanical disruption techniques such as bead beating, grinding with liquid nitrogen (cryogenic grinding), or sonication are frequently employed to physically break open the tough cell walls. However, these methods require careful optimization; excessive mechanical force can shear DNA, leading to fragmented samples unsuitable for some downstream applications. On the other hand, insufficient disruption leaves many cells intact, lowering the overall DNA yield.[8] Enzymatic digestion with lytic enzymes (e.g., chitinase, glucanase) can assist in breaking down the cell wall components but may be costly, time-consuming, or inefficient across different fungal species. The biochemical variability among fungi means that a universal enzymatic cocktail is rarely effective for all samples.[9] The difficulty of effectively lysing fungal cells remains a significant bottleneck in fungal genomic studies. Achieving a balance between thorough cell disruption and preservation of DNA integrity is essential to maximize yield. Continued optimization and combination of mechanical and enzymatic methods tailored to specific fungal species or sample types are critical to overcoming this challenge.

2.4.DNA DEGRADATION CAUSED BY FUNGAL NUCLEASES:

DNA degradation during fungal genome extraction is a significant biological challenge largely due to the activity of fungal nucleases—enzymes that break down nucleic acids. These nucleases are naturally present within fungal cells and play essential roles in DNA repair, recycling, and regulation. However, during cell lysis, nucleases are released into the extraction mixture, where they can rapidly degrade genomic DNA if not properly inhibited, resulting in fragmented or low-quality DNA unsuitable for downstream molecular applications.[10] The problem is exacerbated by the harsh and often prolonged extraction procedures required to breach the tough fungal cell walls. Mechanical disruption, heat, and chemical treatments can inadvertently activate or release more nucleases. Without effective inhibition, these enzymes cleave DNA strands into smaller fragments, reducing both the quantity and integrity of the recovered DNA.[12] To mitigate nuclease-induced DNA degradation, extraction protocols commonly incorporate nuclease inhibitors such as EDTA, which chelates divalent cations (e.g., Mg^{2+} and Ca^{2+}) that are essential cofactors for nuclease activity. Additionally, maintaining low temperatures during extraction—using ice or chilled buffers—helps slow enzymatic reactions. Some protocols also use proteinase K or other proteolytic enzymes to degrade nucleases themselves.[11] Despite these measures, DNA degradation can still occur, especially when working with environmental or wild fungal samples where nuclease activity may be higher or more diverse. Therefore, rapid processing and the use of freshly prepared reagents are crucial. In some cases, adding reducing agents or antioxidants helps protect DNA by neutralizing reactive species that may enhance nuclease activity indirectly.[1] Overall, controlling fungal nuclease activity is vital to preserving DNA quality during extraction. Optimizing buffer composition, temperature, and timing can minimize degradation, ensuring sufficient high-quality DNA for PCR, sequencing, and other genomic analyses.

III. TECHNICAL AND METHODOLOGICAL CHALLENGES:

3.1.SAMPLE PRESERVATION AND QUALITY:

The preservation of fungal and mushroom samples prior to DNA extraction is a critical factor influencing the quality and quantity of genomic DNA obtained. Fungi are highly sensitive to environmental conditions, and improper storage can lead to DNA degradation through enzymatic activity, microbial contamination, or physical breakdown of cellular components. Fresh samples often contain active nucleases and other enzymes that can rapidly degrade DNA if not promptly inactivated.[15] Common preservation methods include freezing samples at ultra-low temperatures (−80°C or in liquid nitrogen), drying using silica gel desiccants, or storing in ethanol or specialized buffers. Each method has its advantages and limitations. For example, freezing effectively halts enzymatic activity but requires continuous cold chain logistics, which may not be feasible in field conditions. Silica drying is convenient for transportation and long-term storage but may cause cellular dehydration that affects DNA integrity. Sample age also impacts DNA quality; older or improperly stored samples tend to yield fragmented or chemically modified DNA that can inhibit PCR amplification and sequencing reactions. Additionally, the choice of tissue type (e.g., fruiting body, mycelium, spores) affects preservation outcomes, as spores generally withstand adverse conditions better than hyphal tissues.[14] Maintaining consistent and appropriate preservation protocols is essential to minimize DNA degradation and contamination, thereby ensuring reproducible and reliable molecular results. Furthermore,

documenting sample metadata—including collection time, storage conditions, and handling procedures—enhances the interpretability and comparability of genomic data across studies.

3.2.INEFFICIENT DNA YIELD AND FRAGMENTATION:

Mechanical disruption techniques such as bead-beating and grinding with liquid nitrogen are commonly employed to break open the tough fungal cell walls. While effective at physically lysing cells, these methods frequently cause DNA shearing, resulting in fragmented genomic DNA. Fragmentation can reduce the suitability of the extracted DNA for applications requiring long intact sequences, such as whole-genome sequencing or long-read technologies.[1] Chemical extraction protocols, including those using cetyltrimethylammonium bromide (CTAB) or phenol-chloroform, are widely used for fungal DNA isolation due to their ability to remove proteins and polysaccharides. However, these methods are time-consuming, involve hazardous chemicals, and often require multiple steps that increase the risk of DNA degradation or shearing. Moreover, improper handling during extraction can exacerbate DNA fragmentation, compromising yield and quality.[18] Commercial DNA extraction kits developed primarily for plants or animal tissues often perform suboptimally when applied to fungi and mushrooms. This is largely due to differences in cell wall composition and the presence of unique fungal contaminants. As a result, such kits may produce low DNA yields or extracts contaminated with inhibitors that negatively affect downstream processes like PCR or sequencing.[30] Studies such as Selosse et al. (2016) emphasize the need for fungal-specific extraction protocols that combine effective mechanical disruption with optimized chemical treatments to maximize DNA yield while minimizing fragmentation. Tailoring extraction methods to fungal species and tissue types is crucial for obtaining high-quality genomic DNA suited for advanced molecular analyses.

3.3.INTERFERENCE WITH ENZYMATIC REACTIONS:

During fungal genomic DNA extraction, co-extraction of compounds such as polysaccharides and phenolic substances often poses significant challenges for downstream enzymatic processes. These contaminants can strongly inhibit the activity of essential enzymes like DNA polymerases and ligases, which are crucial for PCR amplification, restriction enzyme digestion, and library preparation steps required for next-generation sequencing. Polysaccharides increase the viscosity of DNA solutions and interfere with enzyme binding to DNA, while phenolics can bind covalently to nucleic acids or enzymes, altering their structure and function.[28] Despite the inclusion of purification steps designed to reduce these inhibitors—such as ethanol precipitation, column-based cleanups, or the use of specific binding agents—complete removal is difficult, particularly in fungal samples with high levels of such metabolites. This persistent inhibition can lead to weak amplification signals, incomplete digestion, and poor-quality sequencing libraries, thereby compromising the accuracy and reproducibility of genomic analyses (White et al., 2022). Addressing this challenge requires optimized extraction protocols that effectively separate DNA from inhibitory compounds or incorporate additional cleanup procedures tailored to the biochemical complexity of fungal tissues.

3.4.INTERFERENCE WITH ENZYMATIC REACTIONS:

One of the major technical obstacles in fungal genomic DNA extraction is the presence of co-extracted compounds such as polysaccharides and phenolic substances, which can severely inhibit the activity of enzymes crucial for downstream molecular biology applications. These compounds often persist despite DNA purification efforts and negatively affect enzymes like DNA polymerases and ligases, essential for polymerase chain reaction (PCR), restriction enzyme digestion, and library preparation for next-generation sequencing.[26] Polysaccharides, abundant in fungal cell walls and extracellular matrices, tend to co-precipitate with DNA and create viscous, gel-like solutions. This viscosity hinders enzyme access to DNA templates and reduces the efficiency of amplification and enzymatic modifications. Phenolic compounds, on the other hand, may form covalent bonds with nucleic acids or enzymes, altering their conformation and function. These interactions lead to enzyme inactivation or reduced activity, resulting in weak or failed PCR amplifications, incomplete digestion by restriction enzymes, and poor-quality sequencing libraries.[22] Eliminating these inhibitors is challenging because traditional purification methods such as ethanol precipitation, phenol-chloroform extraction, or silica-based spin columns often fail to completely separate DNA from these contaminating substances. Additional treatments using compounds like polyvinylpyrrolidone (PVP), β-mercaptoethanol, or sodium bisulfite are sometimes employed to bind and neutralize phenolics. Nonetheless, the biochemical complexity and variability of fungal metabolites mean that inhibitor removal remains an ongoing issue, especially when working with environmental or wild-collected specimens rich in diverse secondary metabolites.[1] Consequently, developing and optimizing extraction protocols that specifically target these contaminants is critical for improving DNA purity. High-purity DNA free of enzyme inhibitors is essential for reliable PCR, accurate restriction digestion, and successful library preparation, ultimately ensuring robust and reproducible genomic data. Addressing this challenge is vital for advancing fungal genomics and biotechnological applications.

IV. PROTOCOL OPTIMIZATION EFFORTS:

Efficient extraction of fungal genomic DNA requires protocols tailored to overcome the unique biological challenges posed by fungi. Researchers have adapted and optimized traditional methods to improve DNA yield and purity. One common strategy involves extending the incubation period with lytic enzymes such as lysozyme or lyticase, which specifically target fungal cell wall components like β-glucans and chitin. This enzymatic treatment softens the rigid cell wall structure, facilitating better cell lysis and DNA release (Liu et al., 2020). Another important modification is the inclusion of polyvinylpolypyrrolidone (PVPP) or β-mercaptoethanol in CTAB-based extraction buffers. These additives bind phenolic compounds and reduce oxidation, minimizing DNA degradation and improving the purity of the final extract.[24] Post-extraction purification steps have also been enhanced with magnetic bead-based technologies. Such methods efficiently remove contaminants like polysaccharides and secondary metabolites that typically inhibit downstream enzymatic reactions (Gómez-Molano et al., 2021). Furthermore, protocols designed to be compatible with next-generation sequencing (NGS) platforms often incorporate low-salt DNA precipitation and additional spin-column purification steps, which help produce cleaner DNA suitable for high-throughput sequencing (Krishnan et al., 2018).[25] Despite these advancements, achieving consistent results remains challenging because fungal species exhibit vast diversity in cell wall composition and metabolite profiles. Consequently, extraction protocols that work well for one species may not perform equally with another. Thus, continual refinement and species-specific optimization are critical to standardize methods and enhance reproducibility across diverse fungal taxa.

V. EMERGING APPROACHES AND FUTURE PROSPECTS:

5.1.METAGENOMIC AND CULTURE-INDEPENDENT STRATEGIES:

The rise of environmental DNA (eDNA) and metagenomic techniques has revolutionized fungal genomics by reducing the reliance on pure cultures. These culture-independent approaches allow researchers to analyze entire fungal communities directly from environmental samples such as soil, water, or plant material (Tedersoo et al., 2015). By bypassing cultivation, metagenomics captures both culturable and unculturable species, providing a more comprehensive view of fungal biodiversity and ecology. However, these methods face challenges including amplification biases introduced during PCR and difficulties in assembling genomes from complex, mixed-species datasets. Accurate interpretation requires careful bioinformatics analysis and validation to minimize errors.

5.2.NANOPORE SEQUENCING AND MINIMAL DNA PREPARATION:

Recent advances in sequencing technologies like Oxford Nanopore offer the ability to generate long-read sequences from fragmented or lower-quality DNA samples (Ferrarini et al., 2020). This capability partially alleviates the stringent requirement for high-molecular-weight DNA in fungal genome projects.[29] Nanopore sequencing can thus facilitate faster and more cost-effective fungal genome assembly and characterization. Nonetheless, these platforms currently exhibit higher error rates than short-read sequencers, and the associated costs can be prohibitive for some laboratories. Continuous improvements in accuracy and affordability are needed to broaden their applicability.

5.3.STANDARDIZATION OF EXTRACTION PROTOCOLS:

The wide variability in fungal biology necessitates the development of standardized, species- or clade-specific DNA extraction protocols. Such standardization would improve reproducibility and data quality across fungal genomic studies. Integrating these optimized wet-lab protocols with advanced bioinformatics tools can facilitate effective quality control, contamination detection, and accurate genome assembly (Kusari et al., 2021). This alignment between laboratory and computational approaches represents a critical step toward unlocking the full potential of fungal genomics in research and industry.

VI. CONCLUSION:

Extracting genomic DNA from fungi, especially mushrooms, presents numerous technical and biological challenges due to their robust cell wall structures and the presence of various contaminants that hinder DNA purity and downstream applications. Although advances in mechanical disruption techniques and chemical extraction methods have improved DNA recovery, a universal protocol that effectively addresses the diversity among fungal species remains elusive. The incorporation of emerging technologies, along with enhanced sample preservation methods and standardized extraction procedures, offers promising solutions to these persistent difficulties. Moving forward, collaborative efforts across molecular biology, mycology, and bioinformatics will be vital to develop more efficient, reproducible, and scalable approaches for fungal genome analysis, ultimately accelerating research and applications in fungal biology and biotechnology.[12] Fungi, including mushrooms, are a taxonomically rich and ecologically diverse kingdom, with considerable importance

in environmental, industrial, and pharmaceutical contexts. Genomic research into fungi has expanded rapidly, revealing novel biological insights and biotechnological potentials. However, a major bottleneck remains the extraction of high-quality genomic DNA, a prerequisite for successful molecular analyses such as taxonomy, phylogenetics, genome sequencing, and secondary metabolite research.[4] The inherent challenges arise primarily from the complex, multilayered fungal cell walls composed of chitin, β-glucans, mannoproteins, and often melanin, which make cell lysis difficult and reduce DNA yield. Additionally, the abundance of co-extracted inhibitors like polysaccharides and secondary metabolites—phenolics, alkaloids, and terpenoids—pose significant problems by interfering with enzymatic reactions necessary for PCR amplification, restriction digestion, and sequencing library preparation. These biological and chemical obstacles often result in fragmented, low-purity DNA, hampering downstream applications.[19] To overcome these issues, recent advancements have focused on optimizing mechanical disruption methods, refining chemical lysis buffers with additives such as PVPP and β-mercaptoethanol, and employing post-extraction purification technologies like magnetic bead-based cleanups. The development of species-specific and field-compatible protocols has further enhanced DNA extraction outcomes, although fungal diversity continues to challenge universal standardization. Emerging approaches, including metagenomic strategies and culture-independent environmental DNA analyses, offer alternatives to traditional extraction from pure cultures. Meanwhile, sequencing technologies such as Oxford Nanopore are reducing DNA quality constraints by enabling long-read sequencing from fragmented samples. Future progress in fungal genomics hinges on integrating optimized wet-lab protocols with robust bioinformatics tools, establishing standardized extraction methods tailored to fungal clades, and leveraging automation to improve reproducibility, data accuracy, and scalability. Addressing these challenges is crucial to fully harness the biological and biotechnological potential of fungi and mushrooms.

ABBREVIATION TABLE:

S.NO.	ABBREVIATION	FULL FORM
1.	DNA	Deoxyribonucleic Acid
2.	PCR	Polymerase Chain Reaction
3.	CTAB	Cetyltrimethylammonium Bromide
4.	NGS	Next-Generation Sequencing
5.	PVPP	Polyvinylpolypyrrolidone
6.	PVP	Polyvinylpyrrolidone
7.	eDNA	Environmental DNA
8.	RT	Room Temperature
9.	−80°C	Minus 80 Degrees Celsius (used in ultra-low temperature sample preservation)
10.	N_2 (liquid)	Liquid Nitrogen
11.	β-glucans	Beta-glucans (polysaccharides found in fungal cell walls)
12.	β-mercaptoethanol	Beta-mercaptoethanol (used to reduce oxidation during DNA extraction)
13.	CT	Collection Time
14.	P/A	Preservation/Archiving
15.	Selosse et al. (2016)	Refers to the study by Selosse et al., regarding fungal DNA extraction challenges
16.	White et al. (2022)	Refers to White et al., regarding enzymatic inhibition by contaminants

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