Advances in Applied Research on Anaerobic Fungi and Their Plant Cell Wall Degrading Enzymes

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Abstract

Anaerobic fungi possess strong capabilities for degrading plant tissues. Their robust rhizoidal system and a suite of secreted plant cell wall degrading enzymes can efficiently degrade biomass resources into readily utilizable and high value-added compounds. This holds significant practical importance for addressing feed shortages, energy crises, and environmental problems. Based on relevant research, this paper provides an overview of the taxonomic status, research methods, and applied research of anaerobic fungi, offering a reference for further investigation and scientific application of anaerobic fungi and their secreted plant cell wall degrading enzymes.

Full Text

Advances in Research on the Application of Anaerobic Fungi and Their Plant Cell Wall Degrading Enzymes

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Abstract: Anaerobic fungi exhibit strong capabilities in degrading plant tissues. Through their robust rhizoid systems and secretion of a suite of plant cell wall degrading enzymes, they can efficiently convert biomass resources into readily utilizable compounds with high added value. This holds significant practical importance for addressing feed shortages, energy crises, and environmental problems. Based on relevant research, this paper summarizes the taxonomic status, research methodologies, and application studies of anaerobic fungi, providing references for further research and scientific application.

Keywords: anaerobic fungi; plant cell wall degrading enzymes; biomass resources; application research

1 Introduction to Anaerobic Fungi

Anaerobic fungi are important functional microorganisms in the digestive tracts of herbivores that degrade plant cell walls, playing a crucial role in the digestion of plant fiber tissues. They not only utilize rhizoid growth to penetrate the cuticle and lignin of plant cell walls, but also produce highly active cellulases, hemicellulases, esterases, and cellulosomes for degrading plant cell walls [1-2]. The activity of plant cell wall degrading enzymes secreted by anaerobic fungi exceeds that of the commercially used strains *Trichoderma reesei* and *Aspergillus nidulans* [3], offering broad application prospects in bioenergy, feed industry, and biogas fermentation.

Currently, screening for anaerobic fungi with high plant cell wall degrading enzyme activity has become a research hotspot, while utilizing anaerobic fungi and their enzymes to improve biomass resource utilization efficiency holds important practical significance. Therefore, this paper summarizes the taxonomic status, research methods, and application value of anaerobic fungi and their plant cell wall degrading enzymes based on relevant studies.

Due to long-term limited understanding of the morphological and physiological-biochemical characteristics of rumen anaerobic fungi, their biological classification remained inaccurate, and they were once mistakenly identified as rumen protozoa. Until 1975, British scientist Orpin discovered that three types of zoospores from sheep rumen, including *Neocallimastix frontalis*, shared morphological similarities with aquatic chytrid fungi, and their cell walls contained chitin—a characteristic component of fungal cell walls—first confirming the existence of anaerobic fungi in the rumen [4-5]. They were subsequently classified under the fungal kingdom, subdivision Mastigomycotina, class Chytridiomycetes [6]. In recent years, numerous studies have revealed that anaerobic fungi possess distinct morphological and physiological-biochemical features in multiflagellated zoospores, ultrastructure, and hydrogenosomes that differentiate them from other chytrids [7]. With the development of ribosomal DNA (nrDNA) (18S, 5.8S, and 28S) phylogenetic analysis, anaerobic fungi have been reclassified into the phylum Neocallimastigomycota, class Neocallimastigomycetes, order Neocallimastigales [8-9].

After more than 40 years of research, scholars worldwide have isolated over 20 species of anaerobic fungi from the digestive tracts and feces of herbivores (Table 1) [10]. Currently, classification is generally based on morphological characteristics including zoospore flagella number, rhizoid morphology, and thallus features, dividing them into six genera: *Neocallimastix*, *Piromyces*, *Orpinomyces*, *Anaeromyces*, *Caecomyces*, and *Cyllamyces* [11-12].

2.1 In Vitro Culture Techniques

Currently, the isolation and cultivation of anaerobic fungi primarily employ the Hungate roll tube technique. First proposed by Hungate in 1950 for studying rumen anaerobic bacteria, this technique involves inoculating anaerobic microorganisms into sealed Hungate tubes filled with CO or N and containing oxygen-free culture medium for selective cultivation [10]. The method has since been continuously improved and remains an effective technique for studying strict and obligate anaerobes.

The culture media commonly used in laboratories are primarily based on Hungate' s medium [13], M10 medium [14], Lowe' s medium [15], and Orpin' s medium [16], with modifications made according to specific research objectives. These media typically contain phosphate buffer, cell-free rumen fluid, L-cysteine hydrochloride, and resazurin indicator, which not only provide a stable pH environment (6.5–6.7) and rich nutrients for anaerobic fungal growth, but also detect and remove residual oxygen from the medium.

[Figure 1: see original paper] Hungate's anaerobic tubes [10]. (a) Anaerobic tube with liquid culture medium; (b) Anaerobic bottle with liquid culture medium and filter paper strip; (c) Anaerobic tube with solid culture medium.

2.2 Classification and Identification

Since different anaerobic fungal genera exhibit distinct morphological characteristics (Table 2) [8], preliminary morphological identification can be performed under ordinary light microscopes and scanning electron microscopes based on features such as filamentous rhizoids (*Neocallimastix*, *Piromyces*, *Orpinomyces*, *Anaeromyces*) or bulbous rhizoids (*Caecomyces*, *Cyllamyces*), and zoospore flagella number. Additionally, DNA fluorescent dyes can be applied to stain anaerobic fungal nuclei, allowing observation of nuclear distribution under fluorescence microscopy to further classify strains as monocentric (*Neocallimastix*, *Piromyces*, *Caecomyces*) or polycentric (*Orpinomyces*, *Anaeromyces*, *Cyllamyces*) types.

However, culture medium composition, cultivation conditions, and passage number can significantly affect anaerobic fungal morphology, and dynamic changes such as zoospore release and sporangium growth are difficult to observe under laboratory conditions [11-12]. Therefore, classification and identification of anaerobic fungi require combining morphological observation with molecular biological methods. Molecular techniques currently applied for anaerobic fungal identification include 18S rDNA gene sequence analysis, internal transcribed spacer (ITS) sequence analysis, and large-subunit ribosomal DNA (nrDNA-LSU) sequence analysis. However, 18S rDNA sequences of anaerobic fungi are highly

conserved (similarity >97%), limiting their effectiveness for analyzing relationships among anaerobic fungi [17]. Compared with 18S rDNA, ITS sequences of anaerobic fungi are relatively stable yet variable during evolution, located in highly variable regions of nrDNA that can reveal genetic variation at inter- and intra-generic levels. Currently, ITS1 and ITS2 sequences have become important molecular markers for anaerobic fungal molecular identification, phylogenetic analysis, and genetic diversity studies [18]. The nrDNA-LSU sequence is also a highly variable region in nrDNA that can reflect evolutionary relationships among genera, closely related species, or populations within a species [19]. Increasingly, 28S rDNA is being used as a DNA fragment for anaerobic fungal molecular identification and phylogenetic analysis [20-21]. Tan et al. [22] found that combining 18S rDNA, 28S rDNA D1/D2 domain, and ITS sequences enables more accurate genus and species identification of anaerobic fungi.

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3 Application Research of Anaerobic Fungi and Their Plant Cell Wall Degrading Enzymes

Anaerobic fungi possess strong capabilities for degrading various plant cell walls and play important roles in agricultural biomass resource utilization. Through their extensive rhizoid systems, anaerobic fungi invade recalcitrant fiber tissues, disrupt plant cell wall structures, and loosen them, making them more susceptible to degradation by other microorganisms and hydrolytic enzymes. Simultaneously, anaerobic fungi secrete highly active cellulases (endoglucanases, exoglucanases, and -glucosidases), hemicellulases (xylanases), esterases (ferulic acid esterases, acetyl esterases, and *p*-coumaric acid esterases), laccases, and cellulosomes [1-2], which synergistically decompose complex cellulose, hemicellulose, and pectin substances. Through the combined action of anaerobic fungi and their degrading enzymes, plant cell walls are efficiently broken down into soluble sugars such as glucose and xylose, enabling their application in feed industry, bioenergy, and other fields.

Anaerobic fungi perform mixed-acid fermentation using different carbon sources as substrates, with primary metabolic products including formic acid, acetic acid, lactic acid, ethanol, H , and CO [23-25]. Methanogens can utilize these metabolic products while eliminating feedback inhibition on anaerobic fungal growth and reproduction, promoting ATP production and thereby enhancing enzyme activity and yield [26-27]. Co-cultivation of anaerobic fungi with methanogens significantly improves their degradation capacity for cellulose and other substrates while producing substantial amounts of methane and acetic acid as fermentation end products [27-29], providing a theoretical basis for anaerobic fungal application in biogas fermentation engineering.

3.1 Applications in Feed Industry and Animal Husbandry

Straw-based roughage contains large amounts of complex plant polysaccharides that are difficult to degrade, generally unusable by monogastric animals and only 20%–30% digestible by ruminants [30]. Cellulases, hemicellulases, and other plant cell wall degrading enzymes can destroy plant cell walls, hydrolyzing recalcitrant polysaccharides into monosaccharides readily utilizable by animals while eliminating the anti-nutritional effects of lignin, cellulose, and xylan, thereby improving the nutritional quality of straw feed and increasing roughage utilization efficiency. Cao et al. [31] continuously cultured the yak rumen fungus *Neocallimastix* sp. YAK11 for 10 days using oat straw, corn straw, rice straw, and wheat straw as feed substrates, finding that the fungus grown on cellulose-rich feed cell wall substrates secreted highly active cellulases and promoted degradation of crop straws. Paul et al. [32] conducted in vitro fermentation experiments with wheat straw as substrate, demonstrating that co-fermentation with bovine anaerobic fungi and rumen microorganisms for 24 hours increased the in vitro degradation rate of wheat straw. Nagpal et al. [33] isolated anaerobic fungi from the guts of elephants, goats, sheep, and other herbivores that exhibited high xylanase, carboxymethyl cellulase, cellobiase, and filter paper cellulase activities, with in vitro fermentation experiments showing that anaerobic fungi could increase feed dry matter digestibility. The efficient degradation of corn straw, wheat straw, and other agricultural biomass resources by anaerobic fungi demonstrates their broad application prospects for alleviating feed shortages in China' s animal husbandry industry and improving roughage utilization.

Furthermore, direct application of anaerobic fungi as additives has gained increasing attention. Thareja et al. [34] showed through in vitro fermentation experiments with wheat straw as substrate that rumen anaerobic fungi could increase the in vitro degradation rate of wheat straw dry matter and neutral detergent fiber (NDF). Adding pure cultured rumen fungi during rice straw silage decreased NDF and acid detergent fiber (ADF) contents while increasing crude fiber degradation rates [35]. Wang [36] inoculated the anaerobic fungus *Piromyces* sp. CN6 into whole-plant corn silage, with results indicating that rumen fungi improved silage fermentation quality and nutritional composition while increasing crude fiber degradation rates. Additionally, under high-roughage dietary conditions, direct administration of rumen fungi increased daily weight gain in fattening cattle and milk production in dairy cows while enhancing rumen volatile fatty acid concentrations, zoospore numbers, and feed utilization efficiency [37-39].

Compared with commonly used cellulase-producing strains, gut fungi secrete cell wall degrading enzymes with higher activity, more complete enzyme systems, and better pH and thermal stability. Wang et al. [40] found that the xylanase secreted by rumen anaerobic fungus *Piromyces* sp. CN6 had an optimal reaction temperature of 50 ℃ and optimal pH of 5.0, remaining relatively stable at 40 ℃ and pH 5.0–8.0; its acetyl esterase had an optimal temperature of 50 ℃ and optimal pH of 9.0, remaining relatively stable at 40 ℃ and pH

5.0–10.0. Chen et al. [41] discovered that rumen fungal xylanase maintained high activity across pH 3.0–11.0, demonstrating promising industrial application potential. Cao [42] found that the ferulic acid esterase activity secreted by *Neocallimastix* sp. YAK11 remained stable across pH 4.0–9.0, while acetyl esterase activity was stable across pH 5.0–10.0. Moreover, after incubating crude enzyme solution in a 50 ℃ water bath for 24 hours, 53% ferulic acid esterase activity remained. Anaerobic fungi secrete plant cell wall degrading enzymes with high activity, where some hydrolases have optimal reaction conditions close to the physiological conditions of animal digestive tracts, maintain high activity across wide temperature and pH ranges, and exhibit good thermal and pH stability—facilitating feed pelletization and storage and demonstrating substantial potential for industrial feed production.

Currently, feed digestibility is primarily improved by adding cell wall degrading enzymes such as cellulases and hemicellulases. However, challenges remain including unstable degradation effects, poor repeatability, large strain variations, and high, unsustainable costs of continuous fungal cultivation. Future efforts should focus on constructing efficient engineered strains using anaerobic fungal genetic resources and optimizing cultivation and enzyme production conditions for industrial-scale production, enabling large-scale application of anaerobic fungal degrading enzyme genes in feed industry as probiotics or enzyme preparations.

3.2 Applications in Bioenergy

Biomass resources are renewable, low-cost, and abundantly available natural energy sources in nature. Utilizing biomass resources for fuel ethanol production has become a hotspot in sustainable energy research. However, low biomass utilization efficiency, high production costs, and low ethanol concentration in fermentation broth limit the sustainable development of bioenergy. Ranganathan et al. [43] found that anaerobic fungus *Orpinomyces* C1A and its secreted plant cell wall degrading enzymes could more directly and efficiently convert pretreated lignocellulosic biomass into soluble sugars and biofuels. The process involves fungal colonization, polysaccharide hydrolysis, and microbial fermentation stages. First, anaerobic fungi proliferate extensively on biomass, using rhizoid growth to disrupt the plant cell wall surface while secreting cellulases, hemicellulases, and esterases. Air is then injected into the fermenter or cycloheximide is added to inhibit fungal propagation, allowing plant cell wall degrading enzymes to continue converting biomass into glucose and xylose, during which large amounts of soluble sugars accumulate. Finally, recombinant *E. coli* K011 converts these soluble sugars into ethanol and other biofuels. Through this powerful rhizoid system and highly active plant cell wall degrading enzymes, anaerobic fungi efficiently degrade lignocellulose into soluble sugars for conversion to biofuels by recombinant *E. coli* K011, achieving an ultimate conversion of up to 14.1% of corn straw to ethanol.

Compared with industrial production technologies, this anaerobic fungal approach offers the advantage of not requiring additional cell wall degrading enzymes, thus reducing costs. Although some biomass is utilized for fungal growth, reproduction, and enzyme production, resulting in relatively low conversion efficiency from biomass to ethanol, this method is significantly more efficient than microbial ethanol production using *Caldicellulosiruptor bescii* strain JWCB033 [44], *Clostridium phytofermentans* ATCC700394 [45], or co-cultures of *Trichoderma reesei* RUTC30 and *E. coli* NV3 pSA55/69 [46]. However, the fungal colonization stage requires strict control of anaerobic conditions and cultivation parameters, which somewhat limits industrial-scale production. Future improvements should focus on strictly maintaining anaerobic fermenter environments, optimizing biomass pretreatment methods, adjusting microbial inoculation quantities, and supplementing limiting degrading enzymes to achieve higher soluble sugar yields, while applying efficient microorganisms (for converting soluble sugars to ethanol) or microbial consortia to obtain more fuel ethanol and improve biomass conversion efficiency.

3.3 Applications in Biogas Fermentation Engineering

Biogas fermentation is an anaerobic process where microorganisms convert organic matter such as straw and manure into biogas, representing an effective pathway for biomass energy utilization. However, straw-based biomass is rich in cellulose and hemicellulose that are difficult to degrade, becoming a rate-limiting factor in biogas fermentation. Studies have shown that adding anaerobic fungi during straw-based biogas fermentation can improve fermentation quality, increase biomass degradation rates, and enhance biogas production [47-49]. Anaerobic fungi promote hydrolysis of straw biomass, increase methanogen populations during fermentation, accelerate straw degradation, and consequently increase biogas yield. Therefore, anaerobic fungi hold broad application prospects in biogas fermentation engineering.

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4 Summary

Due to the strict anaerobic nature of anaerobic fungi, their preservation, transportation, and application require rigorous maintenance of anaerobic conditions, while their stringent requirements for cultivation parameters (pH, temperature, etc.) pose significant challenges for large-scale application. Future research should further improve preservation methods to maintain high activity during storage and transportation. Meanwhile, continued screening for high cell wall degrading enzyme activity anaerobic fungi from the rumens of ruminants (such as yaks and buffaloes) fed long-term high-roughage diets, coupled with enzyme gene mining, construction of efficient engineered strains, and optimization of cultivation and enzyme production conditions, will facilitate large-scale industrial

production and application of plant cell wall degrading enzymes.

Anaerobic fungi possess advantages and characteristics of microbial fermentation, including rapid fermentation speed, high activity and rich diversity of secreted cell wall degrading enzymes, and efficient synergistic action among these enzymes. Highly active plant cell wall degrading enzymes as additives show broad prospects in feed industry, pulp and paper manufacturing, food industry, and other fields. Laboratory studies have demonstrated that anaerobic fungi and their enzymes can improve biomass resource utilization and produce high-value compounds such as xylanase, esterase, methane, fuel ethanol, and acetic acid from inexpensive lignocellulosic substrates. Therefore, anaerobic fungi can be directly and extensively applied in anaerobic fermentation fields such as bioenergy and biogas production, offering vast industrial development potential and significant social importance.

As global population, energy, and environmental issues intensify, the effective development and utilization of biomass resources has become a consensus trend and research hotspot. Utilizing anaerobic fungi and their plant cell wall degrading enzymes to efficiently convert biomass resources into high-value-added compounds represents an important approach to addressing these challenges, with driving effects on society and the economy.

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