How Allergen Extracts are Made—From Source Materials to Allergen Extracts

Fungal raw materials used to produce allergen extracts

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Objective: To review the topic of fungal raw materials used for the production of allergen extracts and the associated challenges and highlight candidate areas for development before standardized fungal allergen extracts can be commercially produced.

Data Sources: A PubMed search was performed using focused keywords and combined with a review of regulatory documents and industry guidelines. Several books on mycology also were consulted.

Study Selections: The information obtained through the literature, books, and industry was scrutinized and combined with personal experience and expertise to write this article.

Results: Fungi are complex ubiquitous organisms on Earth. They are beneficial and detrimental for humans. Fungi can cause hypersensitivity reactions, including types I, III, and IV. The procurement of fungal raw materials to prepare allergen extracts for diagnosis and possible allergen immunotherapy is complex owing to the intrinsic nature of fungi and their complex genome. Allergen manufacturers produce allergen extracts with variable qualitative and quantitative compositions, which can lead to unpredictable clinical outcomes.

Conclusion: The clinician should be aware of the factors responsible for the qualitative and quantitative compositions of fungal allergen extracts and the reasons that currently preclude their standardization. Scientific advances and collaboration and cooperation between allergen manufacturing companies and regulatory agencies are necessary to improve the quality and consistency of fungal extracts. Moreover, clinicians should understand the limitations of currently available fungal extracts.

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Introduction

Fungi are unicellular and/or multicellular heterotrophic, non-chlorophyllic, eukaryotic organisms ubiquitous in the environment. It is estimated that fungi comprise more than 90\% of the biomass on Earth. Fungi are among the most detrimental and beneficial organisms for humans. For example, although fungi are common causes of spoilage of goods and relevant diseases (including infections and hypersensitivity reactions), they are beneficial because they contribute to the decay of organic matter in the ecosystem, the production of foods for human consumption, and the synthesis of products with valuable medical applications. Without fungi, human life likely would not exist.

Fungi have a complex genome, which accounts for the viable genetic mutations responsible for their adaptability to colonize different environmental niches owing to ecological competition under natural conditions. This phenomenon results in the existence of different fungal strains belonging to particular species with selective capacities of synthesizing various components, including allergens.

Products other than allergens synthesized by fungi include \( \beta \)-glucans, ergosterol, extracellular polysaccharides, secondary metabolites, and microbial volatile organic components. Because fungi are ubiquitous in the environment, humans are exposed to these products through inhalation, ingestion, and/or dermal routes. Fungi often are cultured in the laboratory to obtain products with medical applications. The strains used for this purpose are grown using specific and consistent media and conditions precisely to minimize the occurrence of the spontaneous genetic mutations that take place in nature. For example, fungal source materials used to produce allergen extracts ideally should yield particular clinically relevant allergens and contain limited amounts of nonallergenic products and secondary metabolites.

The purpose of this report is to provide the clinician with an overview of various aspects associated with the production of fungal raw materials suitable to prepare commercial extracts, how the extracts are manufactured, and their associated limitations.
**Classification of Fungi**

Fungi belong to 2 independent kingdoms, Eumycota and Chromista, which diverged many million years ago in the history of evolution. Although the Chromista kingdom plays a relevant role in the ecosystem, it is not mentioned in this article owing to its lack of clinical relevance. Approximately 100,000 fungal species have been identified to date. The estimated number of existing species ranges from 1 to 10 million. However, only a few genera and/or species have been extensively investigated as agents causative of hypersensitivity reactions. A consideration about fungal taxonomy is that fungi have sexual (meiotic) and asexual (mitotic) reproduction. Sexual forms result from meiotic cell division and are diploid. Asexual forms result in the production of haploid mitotic spores or conidia, fragmentation of mycelium, fission of somatic cells, and budding. Meiotic and mitotic forms belonging to the same fungal genus and species receive different taxonomic names. One meiotic form can originate more than 1 mitotic form and vice versa. This is perhaps one of the reasons why the number of existing fungal genera and species is overestimated. Historically, the mitosporic forms of fungi were classified as a taxonomic group called “deuteromycetes” or “fungi imperfecti,” but this group is currently referred to as “mitosporic fungi.” The concepts of ana- morph, teleomorph, and holomorph are used in mycology to clarify the reproductive aspects of fungi. Asexual mitotic forms are referred to as anamorphs, sexual mitotic forms as telemorphs, and their combination is known as holomorph. Table 1 lists a few examples of fungi that receive different names depending on their modes of reproduction.

Two types of fungal classification are used, taxonomic and “artificial.” The taxonomic classification of fungi, which comprises the sexual or mitotic forms of these organisms, is based on phylogenetic relations with ranking levels of morphologic and genetic similarity, which includes kingdoms, phyla, subphyla, orders, classes, families, genera, and species. Fungal taxonomy historically has been based on fungal morphology and physiology. Although widely used, the application of molecular biology methods in fungal taxonomy is still under development, and the association of taxonomic relations changes continuously. The most relevant taxonomic groups of fungi for humans are Zygomycetes, Ascomycetes, and Basidiomycetes.

Ascomycetes are the largest group of fungi, which have the various forms of asexual reproduction previously mentioned (mitotic spores or conidia, chain segmentation, and budding). The “artificial” classification of fungi is based on a combination of different criteria such as specific forms of reproduction, morphology, and physiology. This classification categorizes fungi that are phylogenetically unrelated into groups, which receive names familiar to the general public, such as molds, mildews, and yeasts. Mold is a substance and has no plural; it refers to a superficial growth, as seen as dark colonies or filaments; mildews are an anamorphic stage of Ascomycetes formed by simple chain segmentation, often observed by a superficial, flaky, and whitish growth on plant leaves and building materials; yeasts are asexual forms of many Ascomycetes and few Basidiomycetes, which appear as smooth small colonies that proliferate in many substrates, including foods stored in refrigerators.

**Table 1**

<table>
<thead>
<tr>
<th>Asexual reproduction (anamorphic)</th>
<th>Sexual reproduction (teleomorphic)</th>
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</thead>
<tbody>
<tr>
<td>Penicillium spp</td>
<td>Eupenicillium, Cordyceps, Talaromyces</td>
</tr>
<tr>
<td>Aspergillus spp</td>
<td>Emericella, Eurotium, Neosartorya</td>
</tr>
<tr>
<td>Alternaria spp</td>
<td>Pleospora, Leptosphaeria, Lewia</td>
</tr>
<tr>
<td>Cladosporium spp</td>
<td>Venturia, Mycosphaerella, Capronia</td>
</tr>
</tbody>
</table>

**Fungal Ecology**

Fungi have evolved a wide range of strategies to inhabit the world. They feed by secreting digestive enzymes directly into their environment to digest their food and absorb it. When moisture and organic material is plentiful, fungal spores usually germinate and grow as filaments called hyphae, which later become a mass known as mycelium. Fungi also can withstand desiccation and other environmental stresses. When those conditions occur, they can form spores that function as resting or dispersal propagules. Because the conditions necessary for fungal growth typically exist everywhere, fungi are ubiquitous, cosmopolitan organisms in the outdoor environment.

The number of airborne fungal spores can exceed that of pollen 3 orders of magnitude depending on upon meteorologic conditions and other factors, as determined by aerobiological surveys (Fig 1). Fungi are saprophytes, parasites, or symbionts. Table 2 lists some commonly encountered allergenic fungi, their prevalence, and habitat.

Fungi also grow indoors under typical conditions as a result of the activities of building occupants. These activities include practices such as cooking and bathing or showering. The presence of plants and pets in the home, the existence of carpeting and upholstered furniture, cleaning habits, and maintenance of the air conditioning system can affect fungal populations indoors. Often, the presence of fungi and their products indoors is inconspicuous but should be expected. For example, some fungal colonies are often seen on shower and kitchen walls and within refrigerators, and they do not indicate that abnormal conditions exist. However, when excessive moisture is present within a building; for example, as a result of a plumbing leak, a water intrusion incident, or condensation resulting from a malfunctioning air condition system, rapid fungal colonization might occur unless the source of moisture is remediated as soon as possible. The only parameters that can be theoretically controlled to prevent or decrease fungal proliferation indoors are moisture and the time that materials remain damp.

Different fungal genera and species that grow in buildings are classified into 3 groups according to their requirements of low, moderate, or high moisture, which typically occur sequentially in

![Figure 1](image-url)
Fungi can cause different allergic diseases in humans including rhinoconjunctivitis, allergic asthma, hypersensitivity pneumonitis, bronchopulmonary mycoses, fungal sinusitis, and atopic dermatitis. Primary colonizers include species of Alternaria, Cladosporium, and Phoma; and tertiary colonizers comprise species of Stachybotrys, Chaetomium, Trichoderma, and Aureobasidium, among others.

Fungi are used in industry to obtain different products for human use, including medications, products derived from fermentation, enzymes with different applications, and foods. For example, penicillin, produced by *Penicillium chrysogenum*, was the first antibiotic discovered; *Tolypocladium niveum* is used to manufacture cyclopensine; and *Penicillium roqueforti* and *Saccharomyces cerevisiae* are essential to produce cheese and bread, respectively. Enzymes synthesized by fungi (e.g., amylase and several proteases) also are used in industry for various purposes. In addition, and because fruiting bodies produced by some basidiomycetes and ascomycetes are edible, they are cultivated as food sources, for example, the common mushroom (*Agaricus campestris*) and truffles (*Tuber* species). As described in the following section, occupational exposure to fungi can cause immediate- and delayed-type hypersensitivity reactions. Therefore, the clinician should carefully evaluate the occupational history of patients with hypersensitivity reactions to identify potential links associated with exposure to fungi and their products.

### Association of Fungal Exposure With Hypersensitivity Reactions

Fungi can cause different allergic diseases in humans including rhinoconjunctivitis, allergic asthma, hypersensitivity pneumonitis, bronchopulmonary mycoses, fungal sinusitis, and atopic dermatitis. Primary colonizers include species of *Alternaria, Cladosporium*, and *Phoma*; and tertiary colonizers comprise species of *Stachybotrys, Chaetomium, Trichoderma*, and *Aureobasidium*, among others.

Although exposure to fungal products, including allergens and antigens, occurs primarily outdoors, it can occur indoors under particular circumstances. Owing to the description of poorly defined medical conditions attributed to indoor fungal exposure in the early 2000s, the potential medical effects associated with exposure to fungal products, particularly in the indoor environment, have been extensively reviewed.

Allergic rhinitis and asthma have been shown to be induced by different fungal species, most notably by species of the following genera: *Alternaria, Aspergillus, Bipolaris, Botrytis, Cladosporium, Curvularia, Drechslera, Epicoccum, Fusarium, Penicillium*, and *Stemphylium*. Fungal spores are typically smaller than pollen grains, with a size generally smaller than 4 μm compared with an average of 25 μm, respectively. Thus, fungal spores might be more likely to reach the lower airways and induce chronic pulmonary inflammation and asthma, whereas nonfragmented pollen grains tend to

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**Table 2**

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Prevalence and habitat</th>
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<tbody>
<tr>
<td><em>Alternaria alternata</em></td>
<td>cosmopolitan; found frequently in air, soil, and plant debris</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>cosmopolitan; isolated primarily from plants and soil, found on peanuts</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>ubiquitous; peaks during high humidity and warm weather; found often in humidifiers, dehumidifiers, basements, attics, plants, and food</td>
</tr>
<tr>
<td><em>Aureobasidium pullulans</em></td>
<td>cosmopolitan; found in bark of sequoia trees, on plant leaves, and on bathtubs and shower stalls in homes</td>
</tr>
<tr>
<td><em>Bipolaris sorokiniana (Helminthosporium sativum)</em></td>
<td>cosmopolitan; isolated from soil and plants; found more in tropical and subtropical areas</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>cosmopolitan; often found in regions of high humidity; can be found indoors on decaying fruits and vegetable matter; contaminates grapes and contributes to their fermentation to produce wine</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>common; normal flora on mucous membranes of warm-blooded animals;</td>
</tr>
<tr>
<td><em>Cladosporium herbarum</em></td>
<td>ubiquitous; found frequently in air, soil and plant debris</td>
</tr>
<tr>
<td><em>Epicoccum nigrum</em></td>
<td>cosmopolitan; isolated from soil and plants; common on cereals and seeds</td>
</tr>
<tr>
<td><em>Fusarium moniliforme</em></td>
<td>cosmopolitan; isolated from soil and plants; common in silage and agricultural plants</td>
</tr>
<tr>
<td><em>Mucor spp</em></td>
<td>cosmopolitan; isolated from soil and decaying organic material; soil, and dung</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>ubiquitous; found on food products, damp building materials, house dust; spores are often airborne indoors</td>
</tr>
<tr>
<td><em>Phoma betae</em></td>
<td>common; parasitizes sugar beets; isolated from soil, decaying plants</td>
</tr>
<tr>
<td><em>Puccinia graminis</em></td>
<td>common; parasitic; produces rust-colored spores on leaves and stems of primary host (oats, wheat)</td>
</tr>
<tr>
<td><em>Rhizopus stolonifer</em></td>
<td>ubiquitous; causes spoilage of refrigerated foods, particularly fruits and vegetables; found on cereal grains</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>common; common bread yeast (baker's and brewer's yeasts)</td>
</tr>
<tr>
<td><em>Stemphylium solani</em></td>
<td>common; plant pathogen; causes leaf spot on fruits and vegetables, leaf blight in alliums and cotton</td>
</tr>
<tr>
<td><em>Trichophyton mentagrophytes</em></td>
<td>common; causes superficial skin infections in humans and other animals; “athletes foot fungus”</td>
</tr>
</tbody>
</table>

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**Figure 2.** (A) Fungal growth (arrows) on a shower door from activities of the building occupants and (B) growth on a wall as a result of flooding within a building.
remain in the nasal mucosa. Numerous studies showing an association between fungal sensitization and asthma have been published.17–20 Exposure to Alternaria species spores has been long described as the most relevant allergenic source associated with asthma in arid areas of the world.21,22 An association between exposure to Alternaria species and Cladosporium species spores and emergency department admissions for asthma also has been reported.23 In addition, exposure to total spores, ascospores, or basidiospores has been linked to severity of asthma or allergic rhinitis.24,25

Some thermotolerant fungi, most notably Aspergillus fumigatus, can germinate in the lung, where they cause the allergic and infectious manifestations of allergic bronchopulmonary aspergillosis.26 Aspergillus species indeed could be responsible for a spectrum of hypersensitivity diseases including asthma, allergic sinusitis, and hypersensitivity pneumonitis. The simultaneous occurrences of these disorders have been increasingly recognized and might require careful diagnosis. Less frequently, allergic bronchopulmonary mycoses might be induced by Candida albicans, Bipolaris species, Schizopyllum commune, and Curvularia species.27 The ubiquitous presence of fungi in indoor and outdoor environments has made it difficult to identify those directly responsible in causing human diseases. Colonization and presence of multiple fungal species in the upper and lower airways can have a clinical effect without fulfilling all the diagnostic criteria of allergic bronchopulmonary mycoses. Thus, it is still unclear what role these other fungi isolated from respiratory samples play in impairing lung function. Specific immunoglobulin (Ig) E tests can be used to identify the fungi that sensitize and elicit symptoms in patients, but discordant results are common when using nonstandardized reagents. Cross-reactivity among different fungal species also can contribute to confusion among medical professionals. Thus, there is currently no consensus as to which fungi to test for.28

Allergic fungal sinusitis (AFS) or allergic fungal rhinosinusitis has been associated with affected skin sites.39,40 Delayed- and immediate-type hypersensitivity skin reactions can be induced by these dermatophytes. Skin testing, bronchial and nasal challenges with Tonsurans extract, and specific IgE tests can confirm IgE-mediated hypersensitivity to Trichophyton species in most individuals with positive skin test results.41,42

Fungal metabolites, especially proteases and volatile organic compounds, can stimulate an innate tissue response resembling an allergic reaction, especially in damp water-damaged buildings.43,44 It is not clear whether these “aero-irritant” effects are due to specific IgE sensitization or other causes.

### Manufacturing Fungal Source Materials and Extracts

More than 200 fungal allergen extracts are produced and marketed by US-licensed manufacturers. Allergenic source materials used to produce the extracts are derived from well-defined, pure seed cultures from established suppliers such as the American Type Culture Collection (Manassas, Virginia) and the Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands).45 Licensed manufacturers must have a separate facility dedicated for cultivating
and processing fungi. The techniques for cultivation, harvesting, and processing of the fungi can vary among manufacturers, but the general process is analogous (Fig 3). The seed cultures, derived from a specific strain, are grown on a defined growth medium, inspected for identity and purity, and then used to inoculate batch cultures.

The morphologic and biochemical characteristics of each strain used are verified to confirm their identity and purity. Unstable strains are not suitable for use in manufacturing allergen extracts. Strains capable of producing mycotoxins or other secondary metabolites also should not be used; if this is unavoidable, then validated processing steps to remove them need to be used.

The batch culture medium and the growth conditions are defined based on the specific growth requirements of particular fungal species. Culture parameters known to affect the allergenic composition of an individual fungal species or strain include the growth medium, temperature, pH, cultivation time, photoperiod, and aeration. It is common for manufacturers to use conditions that maximize the production of spores and vegetative growth at time of harvest. Each manufacturer uses parameters unique to its licensed process. The optimization of these growth parameters can be difficult because specific allergens can be produced at different stages of the growth cycle and the designated harvest time might not coincide with the maximum yield for each allergenic component.

Before harvesting, the fungus is inactivated, usually with phenol, and a viability test is performed. This is done to ensure the safety of the manufacturing personnel and to prevent contamination of the manufacturing environment. The fungal cells, mycelia, and spores are harvested by separating them from the culture using sieving, filtration, centrifugation, or a combination of these methods. The spent medium, which can contain secreted allergens, also can be harvested. The recovered cellular fraction is processed to produce a final, dried product suitable for subsequent extraction. This can involve homogenization, acetone treatment, drying, and milling. The spent culture is fractionated to remove nonallergenic low-molecular-weight substances including media components. Common techniques used for this purpose include dialysis, diafiltration, ultrafiltration, or tangential-flow filtration. Alternatively, the concentrated culture filtrate can be directly used as the extraction fluid for the cellular fraction or lyophilized and stored separately. In addition to the variation in source materials derived from fungi, the extracts are frequently poorly characterized and are known to contain many undefined components. They also tend to have high concentrations of proteolytic enzymes, which can have a deleterious effect when mixed with other allergen extracts.

Fungal extracts are designated and labeled based on their "weight-to-volume" (w/v) extraction ratio: a 1:10 w/v extract refers to the soluble material recovered after extracting 1 g of dried fungal
source material in 10 mL of extracting fluid. Fungal cell walls can inhibit the elution of relevant cytoplasmic antigens during extraction, but methods used to increase their release (eg, stirring, cell disruption, or homogenizing) also can increase the extraction of nonallergenic components. Additives such as protease inhibitors or polyvinylpyrrolidone can be added to enhance allergen stability during the extraction process, but might not be appropriate for clinical use.

Compared with manufacturing processes used to produce allergen extracts derived from other allergen sources such as pollen, animal dander, mites, insects, hymenopteran venoms, and foods, those used to manufacture fungal extracts are the most variable. This and the paucity of knowledge about the specific allergens derived from such a diverse source have been the major obstacle for the standardization of commercially available fungal extracts used in clinical practice. No studies have been performed to assess the biological reactivity of currently licensed product by quantitative skin testing. Thus, to date, no fungal allergen products have been standardized in the United States. 48

Regulation of Fungal Extracts

National regulatory agencies, especially in the United States and the European Union, provide guidance documents for the manufacturing and quality control of licensed allergenic extracts. 49, 50 These documents address the need for well-defined manufacturing and quality control of licensed allergenic extracts. As regards the European Union, provide guidance documents for the manufacturers, and others for additional evidence. A panel of fungal allergens for diagnostic purposes. In addition, no 2 registered fungal allergens derived from other allergen sources such as pollen, Penicillium species, could lead to improved extract quality and their clinical utility. Alternatively, novel approaches such as the use of purified or recombinant fungal allergens should be investigated to overcome the problems associated with crude extracts.

Ideally, guidelines for the optimal production of fungal raw materials and their associated processing should be developed to improve the quality and consistency of the fungal allergen extracts produced by a given manufacturer. Meanwhile, the clinician should obtain fungal allergen extracts from a single and reliable supplier to maximize consistency to the best possible extent. Fungal extracts, owing to their high protease activity, should not be mixed with other allergen extracts. The clinician also should be aware of the fact that the types and levels of fungal products that individuals are regularly exposed to might be different than those contained in commercially available allergen extracts. Therefore, the clinician should consider the utility of fungal extracts and the interpretation of the associated test results in particular patient populations.

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