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Solid State Fermentation

Research and Industrial Applications

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169
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Editors

Solid State Fermentation

Research and Industrial Applications

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Preface

Solid-state fermentation (SSF) is a well-established technology for cultivation of different microbes on solid substrates without free-flowing aqueous phase. Filamentous fungi, such as *Ascomycota* and *Basidiomycota* species, are the most commonly used microorganisms for this type of cultivation. The fungi grow in nature on pieces of wood, leaves, roots and soil, mostly solid substrates with various moisture contents but no free water. The SSF is an important alternative to suspended fermentation for the production of value-added products such as pharmaceutical drugs, enzymes, organic acids, pigments, biopesticides and flavour supplements. SSF is also applied in bioleaching, biodigestion, bioremediation, biopulping and mushroom cultivation in the last decades.

SSF technology has obvious advantages over suspended fermentation. First of all, SSF processes can utilize solid waste materials such as agricultural residues and wood chips as the primary substrates for the microorganisms. This provides a mechanism to recover resources from the waste materials for sustainable development. In addition, SSF technology combined with tray reactors and/or moving-bed reactors (such as drum reactors) can substantially improve heat transfer of the materials and prevent blocking during the fermentation processes. SSF also has some disadvantages, especially in modelling and scaling up. Determination of microbial cell mass and growth kinetics on solid substrates during SSF processes is particularly difficult. Another important challenge for the SSF is characterization of the substrate consumption during the fermentation processes.

This book presents recent development in SSF technology. Innovative SSF processes have been described in the book for their applications in food processing, enzyme production and biofuel generation. The book includes cultivation of special microbial culture and cells through SSF processes. Technical potential, limitations

and research needs of the SSF technology are also discussed in the book. The book is designed as a reference book for scientists and engineers who are interested in solid-state fermentation.

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Cultivation of Microbes Under Solid-State Fermentation

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Cultivation of Medicinal Mushroom Biomass by Solid-State Bioprocessing in Bioreactors

Marin Berovic

Contents

Abstract Basidiomycetes of various species and their wide range of pharmaceutically interesting products in the last decades represent one of the most attractive groups of natural products in Asia and North America. Production of fungal fruit bodies using farming technology is hardly covering the market. Comprehensive solid-state technologies and bioreactors are the most promising part for fast and large amount of cultivation of medicinal fungi biomass and its pharmaceutically active products. Wood, agriculture, and food industry wastes represent the main substrates that are in this process delignified and enriched in proteins and highly valuable

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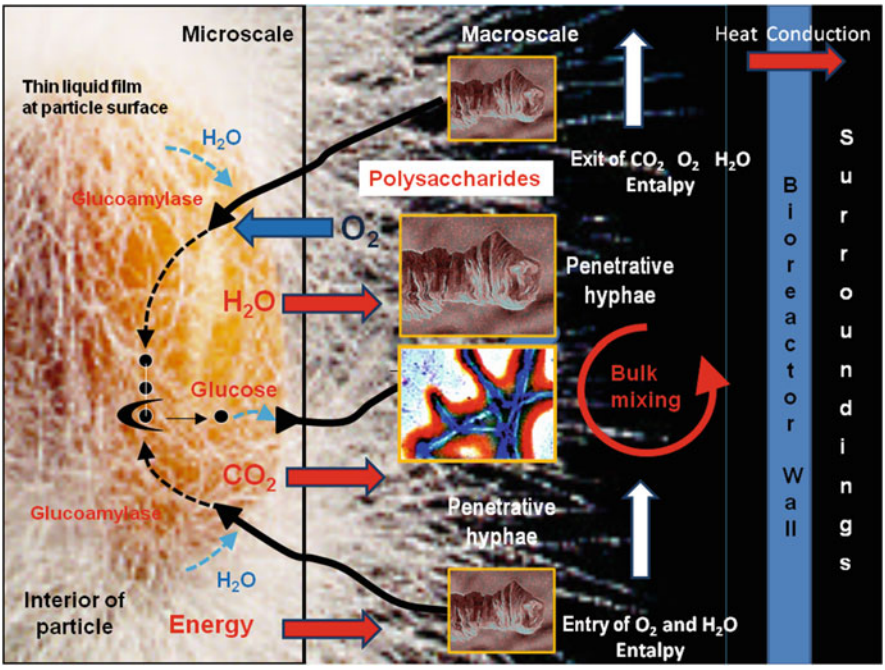
pharmaceutically active compounds. Research in physiology, basic and applied studies in fungal metabolism, process engineering aspects, and clinical studies in the last two decades represent large contribution to the development of these potentials that initiate the development of new drugs and some of the most attractive over-the-counter human and veterinary remedies. Present article is an overview of the achievements in solid-state technology of the most relevant medicinal mushroom species production in bioreactors.

Graphical Abstract



Keywords Fungal biomass, Medicinal mushrooms, Solid-state bioreactors, Solid-state cultivation, Substrates

List of Symbols and Abbreviations

ψ	Water potential
a_w	Water activity
EPS	Exopolysaccharide
M	Water molecular mass
NGF	Nerve growth factor

P	Water vapor pressure
P_0	Water vapor pressure of pure water under the same condition
PSK	Polysaccharide krestin from *Trametes versicolor*
PSP	Polysaccharopeptide from *Trametes versicolor*
R	Gas constant (8.31 J/mol K)
SmB	Submerged bioprocessing
SSC	Solid-state cultivation
T	Absolute temperature

1 Introduction

According to Wasser [1], the number of mushrooms on Earth is estimated at 140,000, yet maybe only 10% of species are known and named. *Basidiomycetes* mushrooms comprise a vast but largely untapped source of new pharmaceutical products in fruit bodies, cultured mycelium, and cultured broth. The practice of using medicinal mushrooms in Chinese traditional medicine dates back into antiquity and has been recorded in ancient Chinese manuscripts [2]. Increased scientific and medical research in recent years published in peer-reviewed journals, especially in Japan, Korea, and China and more recently in the USA, is increasingly confirming the medicinal efficacy and identifying the bioactive molecules [3–5]. Recent advances in chemical technology have allowed the isolation and purification of some of the relevant compounds especially polysaccharides which possess strong immunomodulation and anticancer activities. The bioactive polysaccharides isolated from mushroom fruit bodies, submerged cultured mycelial biomass, or liquid culture broths are either water-soluble β-D-glucans; β-D-glucans with heterosaccharide chains of xylose, mannose, galactose, or uronic acid; or β-D-glucan-protein complexes, i.e., proteoglycans [6]. While many are orally bioavailable, others are mainly effective only by intraperitoneal injection. All of these compounds are currently produced by Asian pharmaceutical companies. These medicinal polysaccharides are primarily modifiers of biological response where these polymers interact with the immune system to upregulate or downregulate specific aspects of the response of the host, and this may result in various therapeutic effects [7]. Their ability to enhance or suppress immune responses can depend on a number of factors including dosage, route of administration, timing and frequency of administration, mechanism of action, or the site of activity. Several of these compounds have been shown to potentiate the host's innate (non-specific) and acquired (specific) immune responses and to activate many kinds of immune cells that are important for the maintenance of homeostasis, e.g., host cells (such as cytotoxic macrophages, monocytes, neutrophils, natural killer cells, dendritic cells) and chemical messengers (cytokines such as interleukins, interferon, colony-stimulating factors) that trigger complement and acute-phase responses [4, 8]. They can also be considered as multi-cytokine inducers capable of modulating gene expression of various immunomodulatory cytokines via specific cell membrane receptors [9, 10]. Lymphocytes governing antibody production (β cells) and cell-mediated cytotoxicity (T cells) are also stimulated.

Mushroom polysaccharides do not attack cancer cells directly but produce their antitumor effects by activating different immune responses in the host. Their mechanisms of action involve them being recognized by several immune cell receptors as nonself molecules, so the immune system is stimulated by their presence. Structurally different β-glucans have different affinities toward receptors and thus generate different host responses [11]. Immunomodulating and antitumor activities of these metabolites are related to immune cells such as hematopoietic stem cells, lymphocytes, macrophages, T cells, dendritic cells, and natural killer cells, involved in the innate and adaptive immunity, resulting in the production of biologic response modifiers [12].

Clinical evidence for antitumor and other medicinal activities come primarily from some commercialized purified polysaccharides that have undergone extensive anticancer clinical trials, such as lentinan from shiitake – *Lentinula edodes*, krestin (PSK polysaccharide-K) and PSP (polysaccharopeptide) from *Trametes versicolor*, grifolan and grifron-D from *Grifola frondosa,* and schizophyllan from *Schizophyllum commune* [1, 11], but polysaccharide preparations of some other medicinal mushrooms also show promising results [13].

2 Solid-State Cultivation

Bioprocessing of various natural sources can be divided generally into processing of liquid – submerged and solid media sources – solid-state cultivation. SSC involves the growth and metabolism of microorganisms in beds of moist solid materials in which the interparticle spaces contain a continuous gas phase and little or no free water. The main sources of water, carbohydrates, phosphorous, nitrogen, and sulfur are intrapartically bounded; therefore the microbial culture applied has to possess the abilities to access the water and essential element sources out the solid matrix [14].

Solid-state cultivation is a three-phase heterogeneous process, comprising solid, liquid, and gaseous phases, which offers potential benefits for the microbial cultivation for bioprocesses and product development. Over the last two decades, SSC has gained significant attention for the development of industrial bioprocesses, particularly due to lower energy requirement associated with higher product yields and less wastewater production with lesser risk of bacterial contamination [15]. Its historical importance for human kind dates from thousands of years ago, mainly for food processing, both in western (bread and cheese) and eastern (koji) countries. Considering the last century and the recent decades, it is used for production of important biomolecules and products for many industries, including food, pharmaceutical, textile, biochemical, and bioenergy, among others [15, 16].

An important advantage of solid-state cultivation over other techniques is that a concentrated product can be obtained from a cheap substrate, such as an agricultural residue with little pretreatment or enrichment. On the other hand, the use of an undefined medium, such as sawdust, might make the product purification more difficult. For this reason, solid-state cultivation seems to be most appropriate for the production of pharmaceutically active animal feed supplements, for which the whole fermented substrate can be used as the product.

In recent years, substantial credibility in employing solid-state cultivation (SSC) technique has been witnessed owing to its numerous advantages over submerged bioprocessing (SmB). In spite of enormous advantages, true potential of SSC technology has not been fully realized at industrial scale [17].

Solid-state cultivation in principal involves the growth and metabolism of fungi in beds of moist solid materials in which the interparticle spaces contain a continuous gas phase and little or no free water. The water activity (a_w) of substrates is an important aspect of fungal cultivation, because the growth of fungi is controlled by the level of a_w. The water activity of a substrate is not the same parameter as its water content but refers to free, unbound, or active water that can support the growth of fungi. The water activity simply represents the ratio of the water vapor pressure (P) in any substrate system to the water vapor pressure of pure water (P_0) under the same condition [18].

$$a_w = P/P_0 \tag{1}$$

Water activity is therefore defined as the equilibrium relative humidity divided by 100, and the scale of a_w extends from 0 (completely dry) to 1.0 (pure water). The water potential ψ can be calculated from the water activity as follows [18]:

$$\psi = RT/M \ \ln \ P/P_0 \tag{2}$$

where R is the gas constant (8.31 J/mol K), T is the absolute temperature, M is the molecular mass of water, and P/P_0 is the water activity.

Water activity has its most useful application in predicting the growth of fungi. It has been established that most fungi cease to grow at water activities below 0.60. This means that the water activity of the substrate determines the lower limit of available water for fungal growth [18].

A major difference between submerged cultivation and solid-state cultivations is therefore the amount of free liquid in the substrate. In the case of submerged cultivation, the amount of dissolved solids rarely exceeds 5–10% by weight (approx. 50–100 g/l), whereas in solid-state cultivation, the solids typically represent between 60 and 80% of the total substrate mass.

The upper limit of moisture content for solid-state cultivations is determined by the absorbency of the solid, which varies between substrates, although for most substrates, a free water becomes apparent before 80% moisture level is reached.

The main mechanism of hyphal growth on surface of solid matrix joins the following steps (Fig. 1):

- Production of fungal polysaccharides on the apical tip of hyphae
- Gluing the apical tip of hyphae on the surface of the lignocellulosic matrix with polysaccharide gel material
- Transferring lignocellulosic enzymes through gel anchorage to the lignocellulosic surface

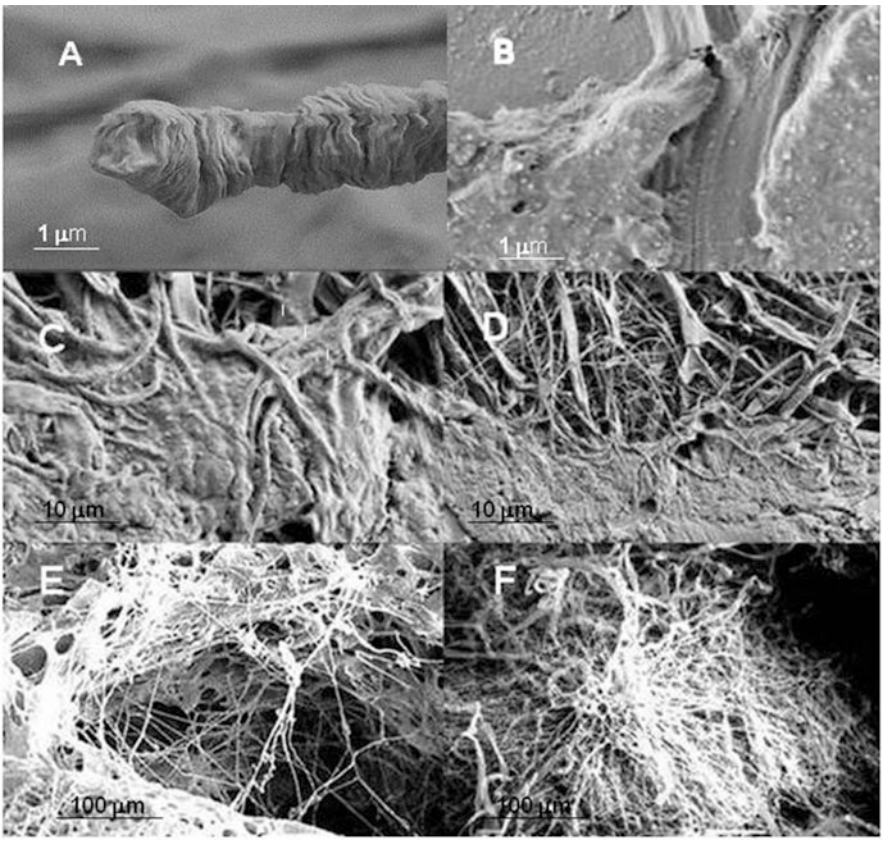


Fig. 1 The main mechanism of hyphal growth on surface of solid matrix. (**a**) Fungal polysaccharides on the apical tip of hyphae, (**b**) cross section of glued apical tip of hyphae on the surface of the lignocellulosic matrix, (**c**, **d**) cross section of fungal growth, (**e**) the void space in overgrown solid matrix, (**f**) fungal mycelia on solid surface [14]

- Depolymerization of lignocellulose and mechanical penetration through the solid matrix

Penetration into substrates is an important phenomenon which has been observed experimentally but has not yet received modeling attention.

3 Differences Between Submerged and Solid-State Bioprocessing

Benefits and the main differences between solid-state and submerged cultivations are summarized in Table 1.

Table 1 Comparison of solid-state and submerged cultivation [14]

Solid-state cultivation	Submerged cultivation
Some products can only be produced well under low moisture conditions. For other products, if the producing organisms require free water, solid-state cultivation cannot be used	A wide range of products can be produced, from a wide range of microorganisms and fungi. Many products are produced best under submerged cultivation
The medium is relatively simple (e.g., grain) and unrefined. It may contain all nutrients necessary for growth or only require wetting with a mineral solution. Pretreatment can be as simple as cooking or grinding. However, the substrate composition and characteristics can be variable	The medium often contains more highly processed ingredients and is therefore more expensive. Unprocessed ingredients may need processing to extract and solubilize the nutrients. With defined media good reproducibility is possible
The low water availability helps to select and protect against growth of contaminants	The water activity is usually very high, and many contaminants can grow well
Media are concentrated and smaller bioreactors can be used, leading to higher volumetric productivities, even when growth rates and yields are lower	Media are dilute and therefore occupy larger volumes, leading to lower volumetric productivities
High substrate concentrations can enable high product concentrations	High substrate concentrations can cause rheological problems. Substrate feeding systems may be required
Aeration requires less power since pressures are lower. Gas transfer is easier since the particles have a large surface area	High air pressures can be required. Gas transfer from the gas to liquid phase is slow and can be limiting
Mixing within particles is not possible, and growth can be limited by the diffusion of nutrients	Vigorous mixing can be used, and diffusion of nutrients is usually not limiting
Ability to remove metabolic heat is restricted, leading to overheating problems	High water content and more dilute nature make temperature control easier
Process control can be difficult due to difficulties in making online measurements and in measuring biomass. The addition of substances during the process is difficult	Many online sensors are available and more are being developed. Additions of substances can be made to control the process
Downstream processing may be simpler since products are more concentrated. However, extracts can be contaminated with substrate components	Downstream processing requires removal of large volumes of water and is more expensive. However, with defined media, product purification may be easier
Liquid waste is not produced	Usually large volumes of liquid wastes are produced
Growth kinetics and transport phenomena have received little attention and are poorly characterized	Much kinetic and transport information is available in literature, which can guide reactor design and operation
Research results and information from the solid-state cultivation can be scaled up or even transferred and applied in liquid-state cultivation	In scaling up fungal submerged cultivation processes, various technical problems need to be solved, such as an increased broth viscosity and oxygen supply
Solid-state cultivation of fungal mycelia is less labor intensive	Submerged cultivation is more demanding and labor intensive

4 Bioreactor Types Used in SSC

SSC bioreactors could be categorized on the basis of the aeration and agitation strategies used. They can be classified as bioreactors without mixing and bioreactors with periodically or continuous mixing. These groups may appear quite different physically but that operate in similar manners. The most applied bioreactors in solid-state cultivation are presented in Fig. 2.

4.1 Tray Bioreactors

Tray bioreactors consist of rooms or cabinets containing a number of individual trays. The cabinet of the bioreactor can be made similar to that of the forced air circulation dryers or it can be in the form of a room built with tiled walls [19]. The characteristics of tray bioreactors are a relatively thin layer of substrate is contained within a tray; the bottom and sides of the tray may be perforated to promote oxygen transfer, carbon dioxide transfer, and released water transfer; at larger scales, a large number of trays are usually stacked in a room; the temperature and humidity of which may be controlled; there is no forced aeration, although air may be circulated around the tray; and the substrate is usually not mixed, although in some cases, the substrate is turned intermittently. The trays holding a 2–5 cm thick layer of the substrate are kept in suitable racks in a cabinet or chamber wherein optimum growth

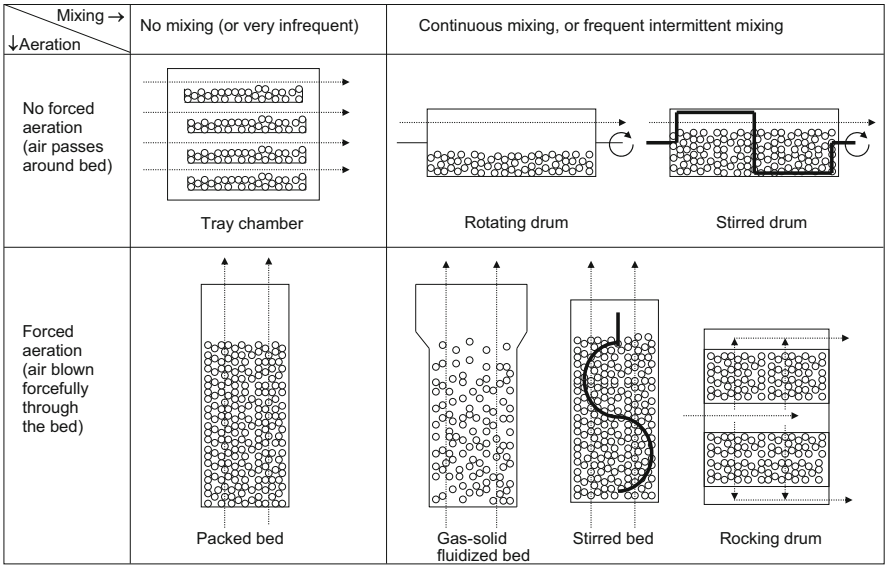


Fig. 2 Classification of SSC bioreactors according to the agitation and used aeration strategies [14]

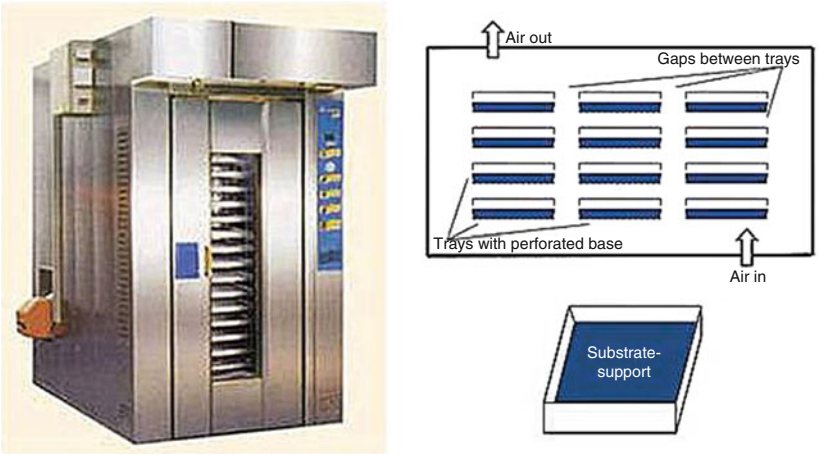


Fig. 3 Tray bioreactor and schematic diagram of inside tray layer and indidual tray [17]

parameters are maintained for obtaining higher yield. The tray chamber is humidified by humidifiers or by forcing in humid air, and provisions are made for the ventilation and control of humidity as well as temperature (Fig. 3).

Heat removal in tray bioreactors is by conduction through the substrate mass to the substrate surface or to the tray itself and then convection from these surfaces to the circulating air. Due to the fact that the air is only circulated around the tray surfaces and not forcefully blown through the bed, mass transfer within the tray is limited to diffusion and heat transfer to conduction. Heat and mass transfer considerations can limit the depth of substrate to as little as 5 cm. With larger depths the inner regions of the substrate may overheat or be deprived of oxygen.

Pleurotus djamor was grown on sunflower seed shell, grape wastes, or potato peels as renewable cheap substrates producing lipases in solid-state cultivation carried out in a tray bioreactor [20, 21].

4.2 Packed-Bed Bioreactors

The characteristics of packed-bed bioreactors are a static substrate mass packed into a column resting on a perforated plate and a forced aeration with controlled temperature and humidity air through the substrate bed, usually coming from below the bed but sometimes from above. Many variations of this basic design are possible, including perforated trays [22, 23]. Very often the design of a packed-bed bioreactor consists of a glass or plastic column with entries at both top and bottom for aeration, while the temperature is controlled either by putting the column in a temperature-controlled room or by passing water through double jackets, fitted around the column. The dominant heat removal mechanism in packed-bed bioreactors depends

on the design and operation of the reactor. Tall thin beds increase the contribution of radial heat conduction. However, for most packed-bed bioreactors, convection is the dominant heat transfer mechanism. During active growth, the air temperature can be lowered to below the optimum temperature for growth, to increase the rate of convective heat transfer, although this only effectively cools the bottom of the bed. If the air has low humidity, then the evaporative cooling becomes significant, although the bottom of the bed tends to dry out. Humidity in the bioreactor can be controlled by using moist air, which can be additionally manipulated to aid in the regulation of the incubation temperature. Several researchers have been successfully using packed-bed bioreactors for a variety of applications [23, 24].

4.3 Rotating Drum Bioreactors

Rotating drum bioreactors are characterized as cylindrical reactors lying horizontally or slightly inclined, with low-pressure air fed into the reactor headspace. They are partially filled with substrate and rotate around the central axis to promote mixing of the substrate, which in some cases might be promoted by baffles. The rotation of the drum is similar to a rotating cylinder in a washing machine – it moves the cultivated solids along the drum. Distinct compartments within the drum are also possible. The main feature of rotating drums is that the air is not forcefully blown through the bed itself. Rather, it is blown through the headspace above the bed, and the gas exchange between the headspace and bed is promoted by mixing of the bed, caused by rotation.

Mixing provided in a rotating drum bioreactor is gentle and among all methods of automated mixing causes the least damage to fungal hyphae and to the agglomeration of substance particles. However, fungi which are highly sensitive to abrasive damage may still be affected. In addition, temperature control is quite difficult, even at small scale, since it is difficult to water-jacket the moving body of the drum, although it is not impossible. These problems increase significantly with scale.

Heat removal from the substrate bed occurs by two main routes: transfer directly to the headspace air by convection and evaporation and transfer through the bioreactor wall by conduction followed by convective cooling of the bioreactor wall. Mixing in rotating drum bioreactors allows the use of dry air to promote evaporation, because water can be replenished by spraying a fine mist of water onto the bed, as it is being mixed. If low humidity air is used for aeration, evaporative cooling contributes to heat removal [25, 26].

4.4 Stirred Aerated Beds Bioreactors

Stirred aerated beds bioreactors fall into the categories of horizontal or vertical stirred bioreactors, depending on the orientation (Fig. 4). Horizontal stirred tank

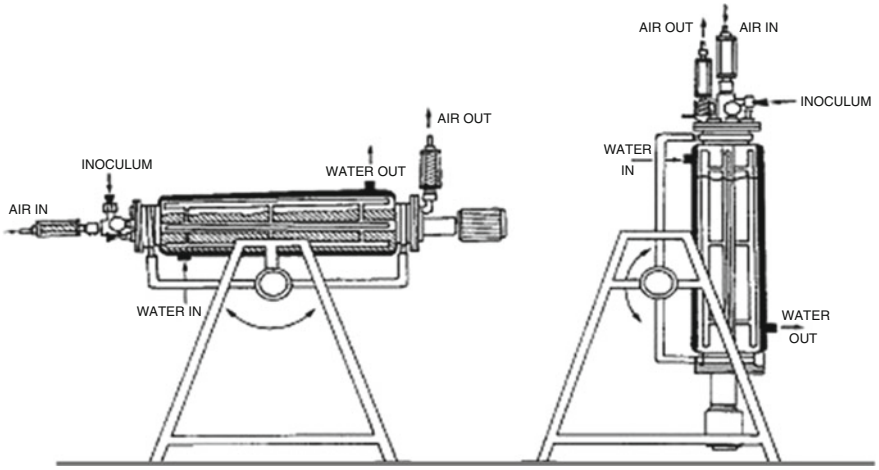


Fig. 4 Horizontal stirred tank bioreactor [27]

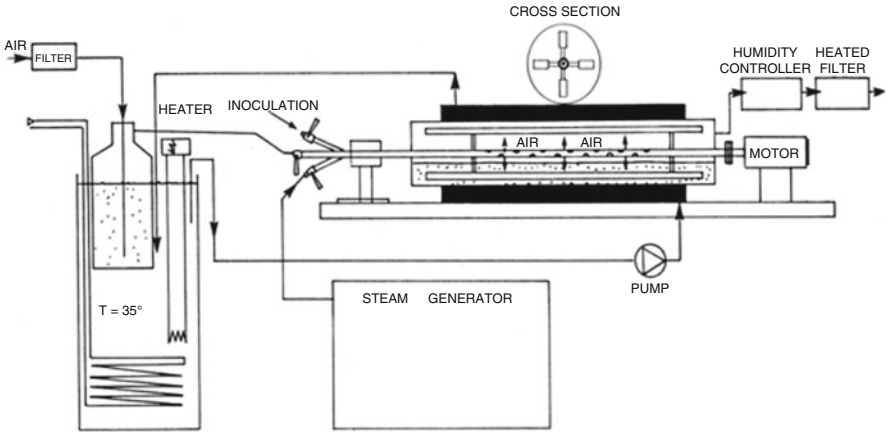


Fig. 5 Horizontal stirred tank reactor and equipment [29]

bioreactors are similar to rotating drum bioreactors, except that mixing is provided by the internal scalpers or paddles attached to a central shaft, rather than by the rotation of the whole body of the bioreactor. Agitation may be continuous or intermittent. Low-pressure air is introduced into the bioreactor headspace [27, 28].

The horizontal stirred tank reactor, presented in Fig. 5, is filled to two-thirds volume with a substrate. Air is introduced through a perforated central shaft embedded in the substrate bed, upon which mixer blades are mounted. Principles of process optimization, such as inoculation, sterilization, mixing, aeration, temperature, and humidity, were studied and optimized in the production of pectinolytic enzymes by *Aspergillus niger* [27]. This type of bioreactor has been later used for solid-state

cultivations of medicinal mushrooms *Ganoderma* sp. [28, 39, 48] and *Grifola frondosa* [29, 42], *Trametes versicolor* [46], and *Hericium erinaceus* [49].

Vertical stirred bioreactors often have similarities with packed beds, since they are usually forcefully aerated. They differ from packed-bed bioreactors by the fact that they are agitated, which may be continuous or intermittent. The advantage of being well mixed helps in avoiding the temperature and moisture gradients, which often occur in packed beds. In the design described by Mitchell et al. [24], three screw agitators are mounted across the breadth of the reactor on a trolley above the substrate bed, with the screws extending down into the substrate bed. The trolley moves backwards and forwards along the length of the bioreactor, such that each location in the bed is intermittently mixed.

5 Substrates for Medicinal Mushrooms Cultivation

In the wild, wood-degrading mushrooms grow primarily on hardwood of trees. However, under artificial cultivation conditions, they thrive on various other substrates containing lignin and cellulose and therefore have a high potential for recycling organic waste materials of different types. As lignocellulose-containing wastes are produced worldwide in large quantities and in many instances they pose a threat to the environment, cultivation of edible and medicinal wood-degrading fungi on lignocellulosic substrates offers almost unlimited possibilities and economically viable potentials for large-scale commercial cultivation on a world scale [30]. Following groups of lignocellulosic substrates have been identified for artificial cultivation (Fig. 6).

Traditional cultivation of fruit bodies on wood logs has been known for centuries. With time, cultivation methods have diversified, modified, and developed (Fig. 7). Besides on wood logs, fruit bodies are being produced on sawdust substrates in trays or beds and in sterilized plastic bags or in bottles. In addition, production of fungal mycelia has been developed in bioreactors, utilizing submerged cultivation technologies in liquid media or solid-state substrates. In order to decrease the cultivation time and to improve the quality, great efforts have been invested into the controlled cultivation in bioreactors. Cultivation of pharmaceutically active biomass of higher fungi in bioreactors with optimized substrate compositions and process parameters enables a shorter cultivation time and a large-scale production under a full process control.

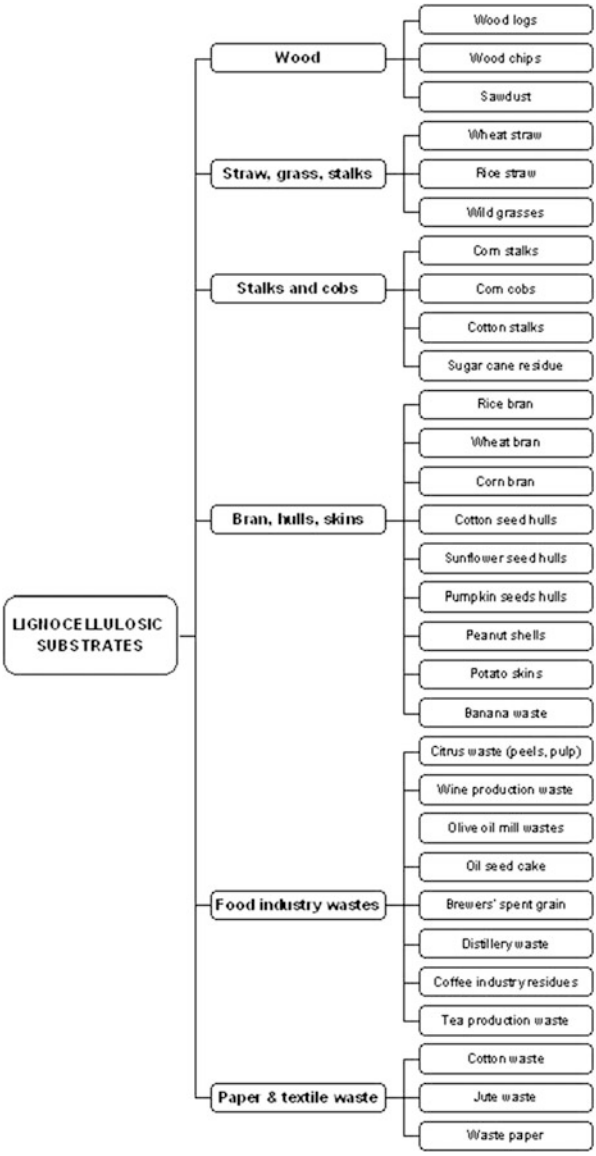


Fig. 6 Lignocellulosic substrates for artificial cultivation of edible and medicinal mushrooms

6 Cultivation of Medicinal Mushroom in Solid-State Bioreactors

An important advantage of solid-state cultivation over other techniques is that a concentrated product can be obtained from a cheap substrate, such as an agricultural residue with little pretreatment or enrichment. On the other hand, the use of an

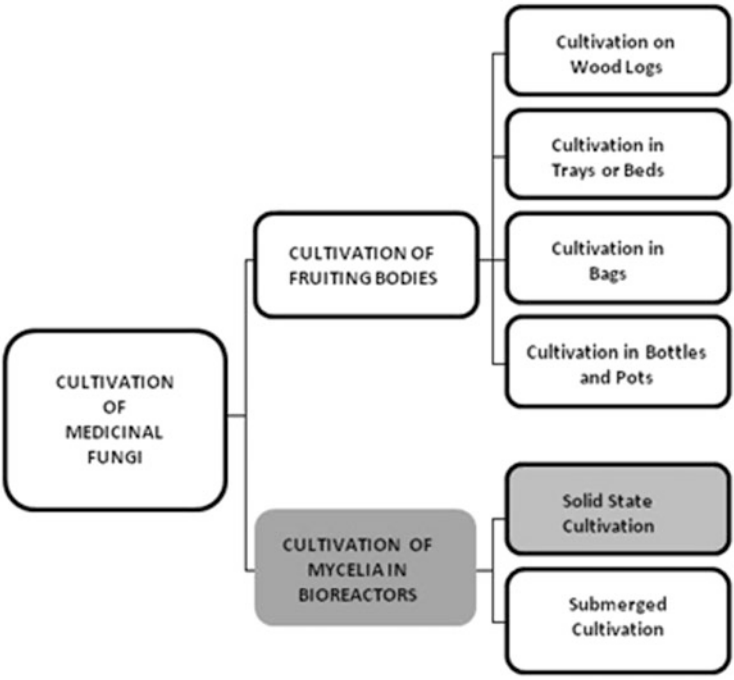


Fig. 7 Technological possibilities of cultivating medicinal fungi fruit bodies or fungal biomass on a commercial scale

undefined medium, such as sawdust, might make the product purification more difficult. For this reason, solid-state cultivation seems to be most appropriate for the production of medicinal mushroom, for which the whole bioprocessed substrate can be used as the product.

Most of medicinal mushroom biomass is still produced using long-term farming technology (e.g., 4 months for *Ganoderma lucidum* fruit bodies and 6 months for *G. frondosa*), compared to 14 days of solid-state cultivation in the first and 18 days in the second case for fungal mycelium type of fungal biomass production that is sufficient for further product isolation. The most important and investigated medicinal mushrooms used in comprehensive cultivation in solid-state bioreactors are *Ganoderma lucidum*, *Grifola frondosa*, *Trametes versicolor*, *Hericium erinaceus*, and *Cordyceps militaris*. SSC of some other species in bioreactors is more in development [1–3].

6.1 *Cultivation of* Ganoderma lucidum

Inoculation density is an important factor for the solid-state cultivation processes. Control of inoculation density was significant for cell growth, morphology, and production of polysaccharides and ganoderic acids [31–33].

An example of a solid substrate composition, used in the production of *G. lucidum* mycelia in a bioreactor, was given by Habijanic et al. [33, 34], consisting of beech sawdust, olive oil, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, and distilled water. During cultivation, the content of extracellular polysaccharides in the solids increased rapidly during the first 7 days, remained relatively constant until 21 days, and then decreased, suggesting that the polysaccharide was actually degraded in the latter stages of the process. The period during which the polysaccharide content decreased corresponded with the time in which the water mass fraction was falling rapidly, from the values of 70–80% that were maintained during the first 21 days to 20% at 35 days [34]. However, it was not clear whether there was a direct cause-and-effect relationship between these two observations.

The feasibility of reusing soy residue for the production of *G. lucidum* was investigated by Hsieh and Yang [35]. The study concluded that soy residue could be successfully reused as a substrate for the solid-state cultivation. However, proper mixing with other solid medium was necessary for adjusting the C/N ratio. Soy residue was found successfully reused as a substrate. However, proper mixing with other solid medium may be necessary for adjusting the C/N ratio. In the solid-state cultivation of *G. lucidum*, the C/N ratio was found to be a crucial factor for the growth rate of mycelium. C/N ratio has to be adjusted in the range of 70–80.

Song et al. [36] used a whey permeate as an alternative additive to a growth medium for the cultivation of *G. lucidum* mycelia in a solid-state cultivation. The optimum condition was found at pH 4.4 and at 29°C. The results showed that the cultivation of *G. lucidum* mycelia could be a potential cost-effective solution for treatment of cheese whey permeates.

Cultivation on a substrate consisting of oak sawdust and corn bran was studies by Montoya et al. [36, 37]. Glucose, soy oil, and yeast extract were found as the best in exopolysaccharide (EPS) production with *G. lucidum* (0.79 g/l EPS). Agitation was found that strongly improved EPS production. The IPS content of the carpophores varied from 1.4% up to 5.5% and 6% in *G. lucidum* and *G. frondosa*, respectively.

For *G. lucidum* solid-state cultivation in pilot-scale bioreactor, Postemsky et al. [38] used a substrate consisting of 26.2% rice straw, 9.4% rice husk, and 1.9% rice bran as well as solid substrate based on 32.5% sunflower seed hulls and 5.0% barley. Substrates were imbibed in 0.5% $CaCO_3$, 2.0% $CaSO_4$ and Cu^{+2}, and enough water to achieve a final moisture of solid matrix of 60%. As an addition of growth stimulants, 1% olive oil was applied. After 2 h pasteurization at 85°C, the substrates were inoculated with grain spawn (8% rate).

In a rare report available by Habijanic et al. [28], the cultivation was carried out in a horizontal stirred tank reactor with a total working volume of 30 l. The conditions were controlled as follows: temperature of 30°C, an airflow of 2 l/min, agitation rate of 80 rpm for 2 min every second day during the first 7 days, and every day during the latter stages of the cultivation. The effect of initial moisture content was evaluated. At least a_w 0.85 was necessary to give satisfactory rates of cell growth and exopolysaccharide production. The control mechanisms of the fungal exopolysaccharide production and consumption were not known; however, the authors suggested that exopolysaccharides served to fasten the hyphae to the surface of the solid particles and to protect the hyphae both from mechanical damage during the agitation and from desiccation at low moisture contents.

Habjanic and Berovic [39] patented a process of growing *G. lucidum* on a solid cultivation substrate using the solid-state cultivation in a horizontal stirred bioreactor. The process enabled a precise leading and monitoring of the fungal growth at sterile conditions. Periodically mixing of 80 rpm for 2 min/day was applied. Production of fungal polysaccharides and fungal biomass on solid substrate based on beech sawdust, olive oil, and mineral salts was studied. Optimal moisture of the solid matrix in the range of 80 to 74% was found. When the moisture content dropped below 57%, the growth of the mycelium and polysaccharide production stopped, but it revived when wet air was applied in further process. Final concentration of the intracellular was 4.5 mg/g and extracellular polysaccharides 1.05 mg/g at biomass concentration of 0.68 mg/g solid substrate. Produced biomass could also be used as a solid inoculum for further cultivation of *G. lucidum* [39].

6.2 *Cultivation of* Grifola frondosa

As the great demand for *G. frondosa* fruit bodies and/or mycelium biomass is in constant increase, cultivation of fruit bodies in bags and biotechnological production of biomass in bioreactors, especially for the production of polysaccharides with medicinal properties, have become the fastest and most efficient ways to respond to the increasing demands. However, several authors report that the quality and quantity of physiologically active substances vary from strain to strain and also depend on location, culture conditions, extraction, and processing procedures [37, 40].

Cheap substrates composed of secondary raw materials, such as agricultural, food, and wood industry residues with little pretreatment or enrichment with mineral salts ($MgSO_4 \cdot 7H_2O$, K_2HPO_4, and $MnSO_4 \cdot 5H_2O$), can be used for *G. frondosa* cultivation. For example, Xing et al. [40] used the substrate consisting of (dry weight basis) 75% of oak sawdust (25% humidity), 23% of corn bran (15% humidity), 1% sucrose (2% humidity), and 1% of calcium carbonate. The moisture content was then adjusted to 62%. Montoya-Barreto et al. [36, 37] assayed two different substrate formulations. The first one consisted of (dry weight basis) 75% oak sawdust (25% humidity), 23% corn bran (15% humidity), 1% sucrose (2% humidity), and 1% calcium carbonate. In the second formulation, 75% oak sawdust was replaced by 50% oak sawdust and 25% coffee spent ground (70% humidity). The humidity was calculated in relation to the components dry weight. It was found that the use of corn

Table 2 Cultivation of *G. frondosa* mycelium by solid-state cultivation in bioreactors

Cultivation	Substrate/inoculation	Cultivation parameters	Yield	Reference
Solid-state cultivation	Wheat bran mixed with tap water 6:4 (w/w), sterilized at 121°C for 40 min, inoculation by mycelium from the agar slants	6–12 days of cultivation, 25°C, relative humidity >97%	5–25% (dry weight of mycelium per dry weight of substrate)	[40]
Horizontal stirred tank bioreactor	2,000 g of milled whole corn plant (*Zea mays*) supplemented with 100 mg $(NH_4)_2SO_4$, 400 mg KH_2PO_4, 100 mg $CaCl_2{\cdot}2H_2O$, 100 mg $MgSO_4{\cdot}7H_2O$, 300 mg $FeSO_4{\cdot}7H_2O$, 4 g $CaSO_4$, 40 ml olive oil, 1,000 ml distilled water, and 500 g of olive press cake	27–40 days, 30°C, airflow 2 l/min. Periodic mixing. Moisture content 80 ± 5%, lowest 50%	Biomass 0.20 mg glucosamine/g solid substrate. Polysaccharides (per g of solid substrate) 30 mg/g extracellular and 14.0 mg/g intracellular	[29]
Horizontal stirred tank reactor, 30 l	Food and wood industry waste materials	38 days, 28°C, aeration 5 l/min, periodic mixing, 5 min/day	Dry biomass 35 g/l; 3.8 mg/g extracellular, 0.70 mg/g intracellular polysaccharides	[41]

grains as substrate for spawn production results an important factor for reducing crop cycle time. A cold shock to 10°C was requisite for basidiome formation. Coffee spent ground was a good substrate for mycelial growth, but not for mushroom production. When using oak sawdust plus corn bran as substrate, consistent yields with combined high biological efficiency (BE) (35.3%) were obtained in crop cycle of 12–14 weeks.

Cultivation of *G. frondosa* mycelium by solid-state cultivation in a bioreactor is still rare. Examples of cultivation parameters are given in Table 2.

6.3 Cultivation of Trametes versicolor

To cultivate *Trametes versicolor*, Knežević et al. [42] were using wheat straw and oak sawdust as carbon sources and 10 ml of modified synthetic medium without glucose, with NH_4NO_3 and pH 6.5, while Stoilova et al. [43] were using wheat bran and oat straw carbon sources. The SSC was carried out using a medium consisted of 4.0 g wheat bran, 2.5 g oat straw, and 2.5 g beetroot press in 300 ml flasks. The moist

of the substrate was adjusted to 60% by mineral salt solution containing 0.14% $(NH_4)_2SO_4$; 0.2% KH_2PO_4; 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2$, $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot 7H_2O$; and 0.002% $CoCl_2$ at pH 4.5.

In *T. versicolor* solid-state cultivation, the modified wheat straw was used by Dinis et al. [44], while de Souza et al. [45] demonstrated that it was possible to produce high laccase levels using the wheat bran as a substrate without any supplementation in the fluidized bed reactor [44, 45].

Rakuš et al. [46] cultivated *T. versicolor* mycelia in solid-state cultivation on a corn straw in 15 l horizontal stirred tank bioreactor. The moisture content of the substrate before inoculation was 2.33 (w/w dry biomass). The inoculum consisted of twenty-five 1 cm^2 cuts mixed in a sterile grinder together with 700 ml of sterilized distilled water. Both inoculation and cultivation were carried out at 24°C and the airflow of 5 l/min. Periodical mixing of 80 rpm, per 2 min (in the first 7 days every second day, and 2 min every day in the last part of cultivation) was used. 5.95 g/l intracellular polysaccharides were isolated.

6.4 *Cultivation of* Hericium erinaceus

The potential of using several agricultural by-products as supplements of sawdust substrate for the production of edible mushroom *Hericium* sp. was evaluated using seven *Hericium* species. All the tested supplements (rice bran, wheat bran, barley bran, Chinese cabbage, egg shell, and soybean powder) were found to be suitable for the mycelial growth of all the tested species. In mycelial growth, soybean powder was the best supplement for *Hericium americanum*, *Hericium coralloides*, and *Hericium erinaceus*, while barley bran was the best for *Hericium alpestre*, *Hericium laciniatum*, and *Hericium erinaceus*. For *Hericium abietis*, rice bran and Chinese cabbage were the best. The possibility of mushroom production on oak sawdust substrate with 20% rice bran supplement was demonstrated with *H. coralloides*, *H. americanum*, and *H. erinaceus*, which showed 26–70% biological efficiency. Our results also showed that strain selection is important to improve biological efficiency and mushroom yield in *Hericium* cultivation [47].

Gerbec et al. [48, 49] cultivated *Hericium erinaceus* in a 15 l horizontal stirred tank reactor (HSTR) of our own construction and design. The cultivation was carried out at 24°C and 5 l/min airflow. Periodical mixing of 80 rpm for 2 min (in the first 7 days every second day, and every day in the last part of cultivation) was used. Although solid substrate was intensively covered by fungal biomass, no erinacine A production was detected. The main reason for this could be the lack of precursors needed for erinacine A biosynthesis. Substrate used did not include enough starch to support the fungal metabolism in sense of erinacine A synthesis. During cultivation carbon dioxide was ventilated out of solid matrix, therefore eliminating its stimulatory effect, possibly affecting erinacine A biosynthesis [49].

Han [50] studied *Hericium erinaceus* solid-state cultivation on cornmeal degrading starch and upgrading nutritional value. On the basal medium which

consisted of cornmeal and salt solution, *H. erinaceus* produced a strong α-amylase on the 15th day after inoculation, which resulted in a 52% degradation of the starch. By supplementation with 5–15 g soybean meal per 100 g cornmeal, the α-amylase activity and degradation rate of starch were raised significantly. Prolongation of fermentation time from 15 to 30 days did not increase significantly the degradation rate of starch, though the α-amylase activity reached its maximum value of 179 U/g on the 20th day after inoculation.

6.5 *Cultivation of* Cordyceps militaris

For the cultivation of *Cordyceps militaris*, substrates from rye seeds and spent brewery grains were mixed in different proportions (9:1, 8:2, 7:3, 6:4, 5:5, 4:6). Water was added to the mixture to achieve 65% moisture content and 100 g of substrate weight. Spent brewery grains represent a readily available, low-price substrate for cordycepin solid-state production. So far the highest reported concentrations (10.42 mg/g) obtained on solid-state substrates were reported [51].

7 Conclusions

Reports on pharmacological activity of solid-state bioreactors produced *G. lucidum* biomass and extracts, partly purified and isolated compounds from fungal mycelia biomass are convincing. There is abundant scientific evidence that triterpenoids, polysaccharides, and proteoglycans produced in solid-state bioreactors have been effective also in in vitro and in vivo testing [10, 11]. Synergistic effects of mixtures of active components have been known, however, but their biological activities need further assessment before they can be fully accepted not only by the traditional Asian medicine but also by the Western science and medicine. In this respect, modern biotechnological cultivation methods in solid-state bioreactors enable fast, efficient, and economical production of *G. lucidum* biomass in sufficient quantities for potential future pharmaceutical large-scale industrial production [39, 48].

G. frondosa can be regarded as a natural bioreactor for the production of healthy nutritious food and pharmaceutical compounds with beneficial effects. The beauty of its nature is that it is a gourmet and medicinal mushroom, willing to grow on simple cellulose and lignin containing substrates, including waste materials from agricultural production, as well as on liquid substrates in bioreactors, where its hyphae excrete medicinal polysaccharides into the medium. Isolation is rather simple and is based on precipitation with ethanol. Crude extracts show equal or stronger pharmacological activity as purified compounds, which suggests potential synergistic effects of several naturally occurring compounds. Expensive high-tech purification procedures for isolation of pure pharmaceutical substances may therefore not be needed [29, 42]. However, from the viewpoint of Western science and medicine and

pharmaceutical legislation and regulations, this might be one of the main obstacles hindering the introduction of *G. frondosa* products as registered pharmaceuticals. In any case, further research is needed to fully understand all mechanisms of pharmaceutical effects and to identify potential side effects of *G. frondosa* medicinal preparations [52].

Convincing reports on in vitro and in vivo pharmacological activity of extracts, partly purified preparations and isolated compounds from SSC medicinal mushroom biomass as terpenoids and polysaccharide krestin (PSK) and polysaccharide-peptide (PSP) of *Trametes versicolor* [43, 46] also with immunomodulation, antibody production activities. In vitro and in vivo studies PSK and PSP shown as pure substances and also as PS mixture shown of both highly affecting immune cell proliferation and highly express antitumor activities [46, 53–58].

Among the compounds isolated from *H. erinaceus* fruit bodies and cultured mycelia are polysaccharides and diterpenoids where the most interesting are the low-molecular-weight compounds belonging to a group of cyathin diterpenoids (erinacines A–K, P, and Q) [59–61]. Several of them, i.e., erinacines A–H, are known to have a potent stimulating effect on nerve growth factor (NGF) synthesis in vitro [59–65].

Great potential of pharmaceutical active compounds and *Cordyceps militaris* extract contains many biological bioactive materials, such as the terpenoids cordycepin and cordycepic acid, polysaccharides, sterols, and other compounds [66]. *Cordyceps militaris* main active component is terpenoid cordycepin that inhibit the development of cancer cells including antitumor, anti-metastatic, insecticidal, antiproliferative, antibacterial, antileukemia, and antimalarial activities. The second main active components are polysaccharides, which research have shown to be effective in regulating blood sugar and also have anti-metastatic and antitumor properties [66–68].

Although solid-state cultivation of fungal biomass in bioreactors does not produce fungal fruit bodies, its products also represent great potential of delignified and in proteins enriched biomass with various highly valuable pharmaceutically active compounds that could be extracted and isolated or simply pulverized and applied in veterinary use. In this respect, modern biotechnological cultivation methods in bioreactors enable fast, efficient, and economical production of medicinal fungi biomass in sufficient quantities that could be applied in pharmaceutical large-scale industrial production.

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Design and Operation of a Pilot-Scale Packed-Bed Bioreactor for the Production of Enzymes by Solid-State Fermentation

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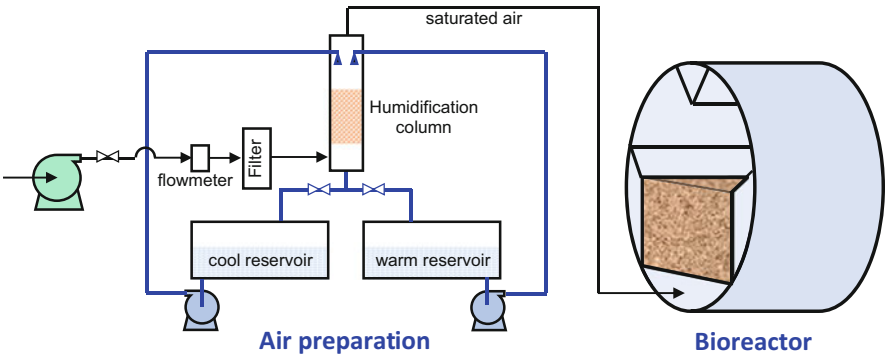
Abstract In this review, we describe our experience in building a pilot-scale packed-bed solid-state fermentation (SSF) bioreactor, with provision for intermittent mixing, and the use of this bioreactor to produce pectinases and lipases by filamentous fungi. We show that, at pilot scale, special attention must be given to several aspects that are not usually problematic when one works with laboratory-scale SSF bioreactors. For example, it can be a challenge to produce large amounts of inoculum if the fungus does not sporulate well. Likewise, at larger scales, the air preparation system needs as much attention as the bioreactor itself. Sampling can also be problematic if one wishes to avoid disrupting the bed structure. In the fermentations carried out in the pilot bioreactor, when the substrate bed contained predominantly wheat bran, the bed shrank away from the walls, providing preferential flow paths for the air and necessitating agitation of the bed. These problems were avoided by using beds with approximately 50% of sugarcane bagasse. We also show how a mathematical model that describes heat and water transfer in the bed can be a useful tool in developing appropriate control schemes.

Graphical Abstract



Keywords Inoculum preparation, Intermittent mixing, Mathematical modeling, Monitoring and sampling, Pectinases and lipases, Scale-up

1 Introduction

This review describes our experiences with building and operating a pilot-scale packed-bed bioreactor for solid-state fermentation (SSF). It describes why we chose this particular bioreactor design and our most recent results. Our aim is to provide some insight into the thinking behind the design and operation of the bioreactor and some of the key challenges that we faced; this type of information is often not given in scientific papers. We hope that this description of our experiences will be useful to other researchers intending to scale up their laboratory-scale SSF bioreactors to pilot scale.

2 Choice of Bioreactor Type and Design Features

2.1 Available Bioreactor Types

When we decided to build a bioreactor with a capacity for several kilograms of solid substrate, the first decision was which type of bioreactor to build. Bioreactor types for SSF can be classified into four groups based on how the bed is agitated and how the bed is aerated [1]:

1. Tray-type bioreactors – the bed remains static or is mixed infrequently (e.g., once or twice per day) while air is circulated around the bed, not being forced to flow through it.
2. Packed-bed bioreactors – the bed remains static or is mixed infrequently (e.g., once or twice per day), with air being blown forcefully into the bed, having to flow through the bed in order to leave it.
3. Rotating- or stirred-drum bioreactors – the bed is agitated continuously or frequently in a horizontal drum, with air being circulated through the headspace above the bed, not being forced to flow through the bed itself.
4. Forcefully aerated agitated bioreactors – the bed is agitated continuously or frequently, with air being blown forcefully into the bed, having to flow through the bed in order to leave it.

2.2 Choice of a Packed-Bed Bioreactor with Intermittent Agitation

We considered several factors in choosing a packed-bed bioreactor with provision for intermittent agitation. The main consideration was that forced aeration would be necessary in order to allow effective removal of waste metabolic heat from the bed. This left the choice between a packed-bed bioreactor and a forcefully aerated

agitated bioreactor. The choice between these two bioreactor types was based on considerations as to the frequency with which the bed should be mixed.

Two thoughts guided our decision to provide for intermittent agitation. Since most of our work involved the cultivation of filamentous fungi, we wanted to leave the bed static most of the time. Although some filamentous fungi can tolerate continuous agitation, agitation significantly affects the growth morphology, with aerial hyphae being squashed into a moist biofilm at the particle surface. A static bed allows aerial hyphae to grow into the inter-particle spaces, where they are surrounded by an air phase [2]. However, we were also aware that it might be necessary to mix the bed several times during the fermentation. Mixing might be necessary to enable the addition of water to the bed or to prevent the formation of agglomerates and the related phenomenon of channeling.

Addition of water is often necessary because the forced aeration in a packed bed tends to dry the bed out, even if saturated air is supplied at the air inlet. This occurs because the air removes metabolic heat from the solid particles as it passes through the bed: the resulting increase in the air temperature increases its water holding capacity, creating a driving force for evaporation [3]. This evaporation can cause the water activity of the solids in the bed to fall to values that cause a significant decrease in the rate of growth and product formation, meaning that it becomes necessary to replenish the water. It is not feasible to add water uniformly to a static bed; rather, the water should be sprayed as a fine mist onto the surface of the bed as it is agitated.

Channeling occurs if cracks appear in the bed or the bed pulls away from the walls, allowing the air to flow preferentially though the gaps, rather than through the bed itself. The appearance of cracks or gaps is common in SSF processes with filamentous fungi, resulting not only from a decrease in particle size as the bed dries and as the solid particle shrinks as it is consumed by the fungus but also from the fact that the fungus tends to bind the particles together into agglomerates, promoting shrinking of the bed as a whole [3]. When channeling occurs, it is necessary to agitate the bed, with the intention of breaking agglomerates and allowing the bed to be "reseated," with a layer of substrate particles of uniform height across the whole bed. It should be noted that agitation is not always effective at breaking agglomerates, such that it can be appropriate to undertake "preemptive" mixing events, with the intention of preventing channeling from occurring in the first place [4].

2.3 Bioreactor Designs that Were Considered but Discarded

We considered several designs before settling on the packed-bed design. Two key designs that were carefully considered, but later discarded, were a horizontal stirred drum with a perforated bottom and a horizontal cylinder with air provided through perforated walls and removed through a perforated pipe at the central axis (Fig. 1).

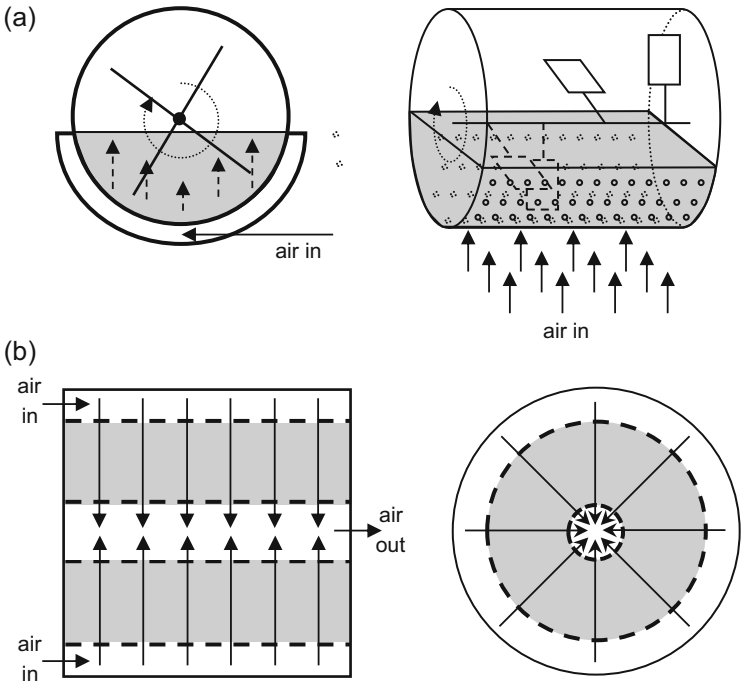


Fig. 1 Bioreactor designs that were considered but were discarded. (**a**) A horizontal stirred drum with a perforated bottom. In the diagram on the right, the air chamber has been removed in order to make the perforated bottom of the cylinder visible. (**b**) A horizontal cylinder with air provided through a perforated outer wall and removed through a perforated pipe at the central axis. In both cases, substrate bed is indicated in gray. Part (**a**) is reprinted from Mitchell et al. [3] with the kind permission of Springer

The horizontal stirred drum with a perforated bottom was discarded because the bed is not of a uniform height, due to the circular cross section of the drum (Fig. 1a). During static periods of operation, the air would tend to flow preferentially through the shallower parts of the bed; there would be little or no flow through the deeper parts of the bed. In such a bioreactor, the whole bed would only be aerated effectively if the bed were mixed continuously.

The horizontal cylinder with perforated walls and a perforated pipe at the central axis had the potential to ensure uniform airflow through the bed (Fig. 1b) but was discarded due to potential complications in design and operation. The first complication is that, in order to avoid preferential flow through some parts of the bed, such a bioreactor would need to be always full, to ensure that the flow paths through the bed, from the wall to the center, were of equal length. We considered that it might be possible to divide the "air jacket" between the outer wall and the substrate bed into compartments. It would then be possible to close off some compartments if the bioreactor were used when only partially full, ensuring that air were only introduced into the bed itself. However, it soon became clear that the engineering would be

unnecessarily complex. The second complication is that, with the cylinder being operated completely full of substrate, it would be difficult to mix the bed if it were necessary to add water or to reseat the bed after the appearance of cracks or gaps.

Having decided on a traditional packed bed, with the solid bed resting on a perforated base plate, the next decision was whether or not to have internal heat transfer plates, such as those used in the Zymotis bioreactor [5]. Simulations with a mathematical model of this type of bioreactor had shown that, for the heat transfer plates to be effective in removing the metabolic heat generated by a fast-growing fungus, it would be necessary for the plates to be about 6 cm apart [6, 7]. Such a close spacing would make it difficult to mix the bed if it were necessary to add water or to reseat the bed after the appearance of cracks or gaps.

2.4 *Aspects of the Final Design of the Bioreactor that Was Built*

The final design (Fig. 2) was a traditional packed bed, constructed in stainless steel (AISI 306). The bed height can be varied as desired, up to a maximum of 50 cm, by loading more or less substrate into the bed chamber. Since this bed chamber is rectangular, the bed height is uniform, even if the bioreactor is not used at its full capacity of 210 L (corresponding to a base of 70 cm by 60 cm and a bed height of 50 cm, Fig. 2b).

The question then arose as to how the intermittent agitation of the bed should be done. Two potential designs were considered but discarded. First, a rotating agitator could be inserted into the bed. Such a design has been used in the bioreactors of INRA Dijon, with the use of traveling screw augers (which mix by lifting the substrate and allowing it to fall) (Fig. 3a) or a non-traveling planetary-type mixer (Fig. 3b) [8]. Second, either rotating agitators or static blades could remain in the same position, with the whole bed being rotated, such as done by the group of PUC in Chile (Fig. 3c) [9]. However, for these designs involving mechanical agitators in the bed, it would be desirable to remove the agitator from the bed after the agitation events, in order to avoid preferential air paths next to the stopped agitator during static operation. This would have required careful mechanical design and significantly increased the cost of our bioreactor. In our final design, the bed was mixed by rotating the whole bioreactor around its central axis, enabling mixing without the insertion of moving mechanical parts within the bioreactor itself. The bioreactor rotation was achieved with an electric motor placed outside of the bioreactor. The V-shaped wedge protruding into the headspace (Fig. 2a) was intended to help break up any agglomerates of substrate particles during the rotation. It should be noted that the bed height is not necessarily uniform after an agitation event, but it is an easy matter to open the lid at the top of the bioreactor and then flatten the top of the bed manually.

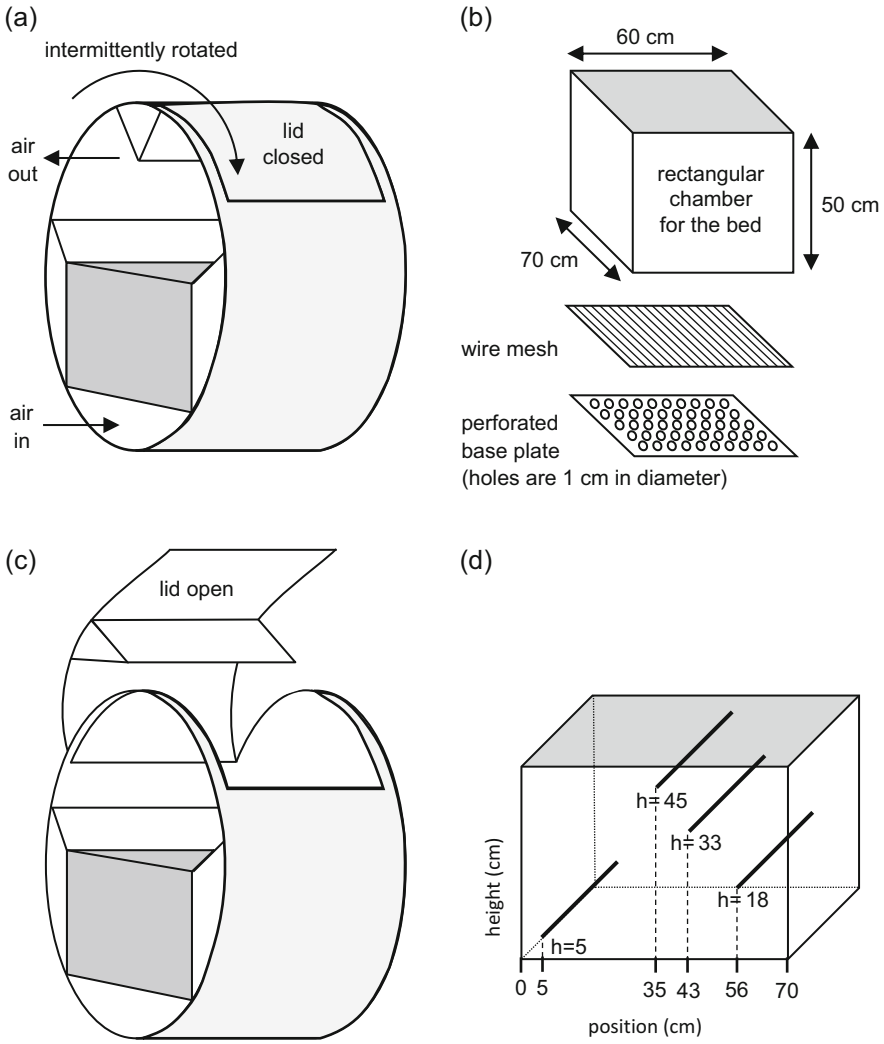


Fig. 2 Details of the pilot bioreactor. (**a**) Perspective of the bioreactor, with the lid closed. The circular front face has been removed so that the bed chamber is visible (in darker gray). The bed is agitated by rotating the whole bioreactor around its central axis. (**b**) Exploded view of the substrate bed chamber. The wire mesh prevents small particles from falling through the 1 cm diameter holes in the base plate. (**c**) Perspective of the bioreactor, as in (**a**) but with the lid open, allowing not only loading and unloading of the bioreactor but also the removal of samples from the top of the bed during the fermentation. (**d**) Positions of the thermocouple sleeves in the bed chamber. In this diagram, the front face of the bed chamber is the same face that is visible at the left in (**c**) (i.e., the bed chamber has been rotated slightly counterclockwise). Each sleeve contains four thermocouples at different horizontal positions. Part (**a**) is adapted from Mitchell et al. [3] with the kind permission of Springer

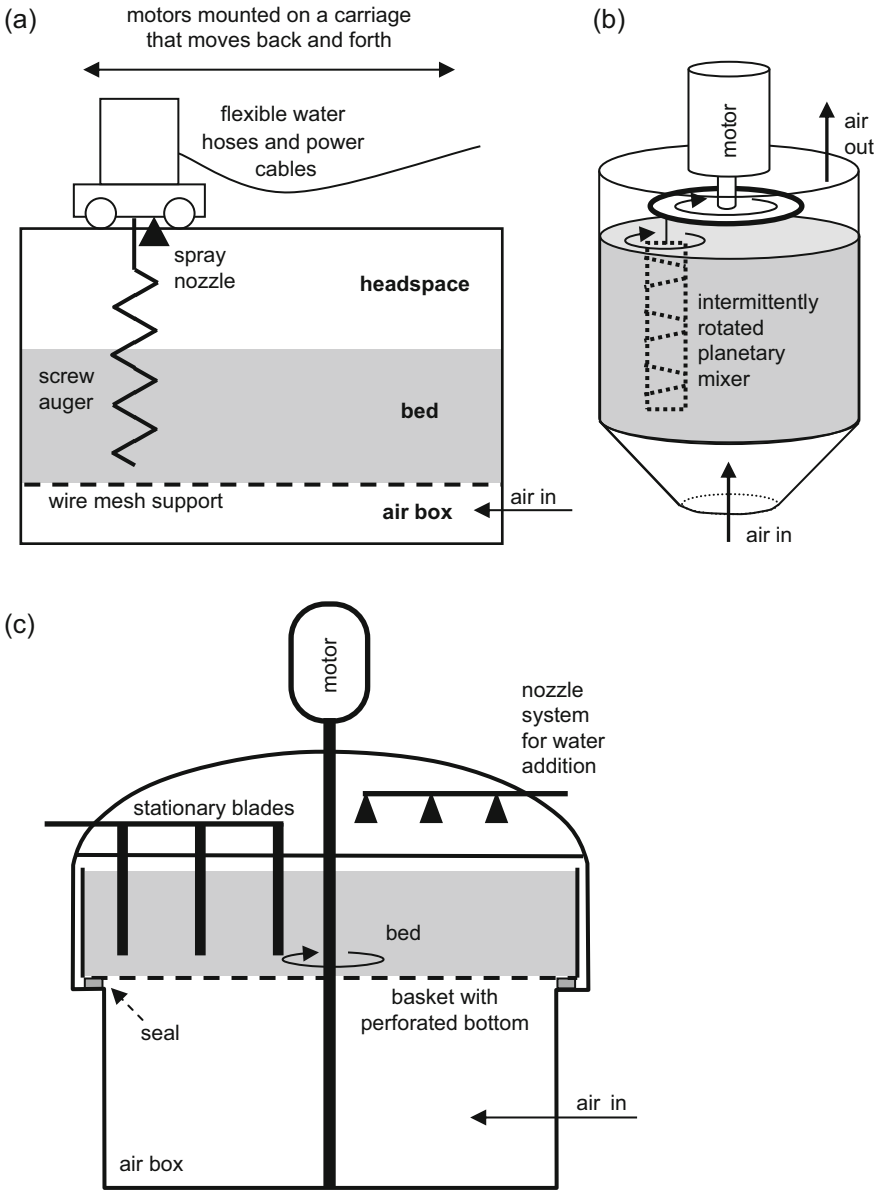


Fig. 3 Potential ways of providing intermittent mixing in a packed-bed bioreactor that were considered but discarded. (**a**) A system of traveling screw augers [8]. (**b**) A planetary mixer [8]. (**c**) A bed that moves past mixers that remain in place [9]. Parts (**a**), (**b**), and (**c**) are adapted from Mitchell et al. [3] with the kind permission of Springer

Cost considerations also affected the design of the aeration system. We considered passing the inlet air pipe through the central axis of the bioreactor. This would have allowed for the possibility of aeration during mixing, such that the bioreactor could operate somewhere between a rotating drum (the third bioreactor group listed in Sect. 2.1) and a forcefully aerated agitated bioreactor (the fourth bioreactor group listed in Sect. 2.1). However, introducing air through the central axis would have significantly increased costs.

2.5 *Design of the Air Preparation System*

Given the importance of the forced aeration in removing the metabolic heat from the bed in a packed-bed bioreactor, the temperature, humidity, and flow rate at which the air will be supplied are key considerations [3, 10]. Our air preparation system is shown in Fig. 4.

The airflow rate is not difficult to control: if the compressor or blower is designed to supply a high flow rate, then a butterfly valve can be used to regulate the flow. Although such a valve could be controlled automatically, for example, in response to temperatures measured in the bed, we manipulated our butterfly valve manually.

We used a blower that was already available to us. It was capable of giving volumetric airflows of the order of 150 $m^3\ h^{-1}$. Based on our bioreactor design, this

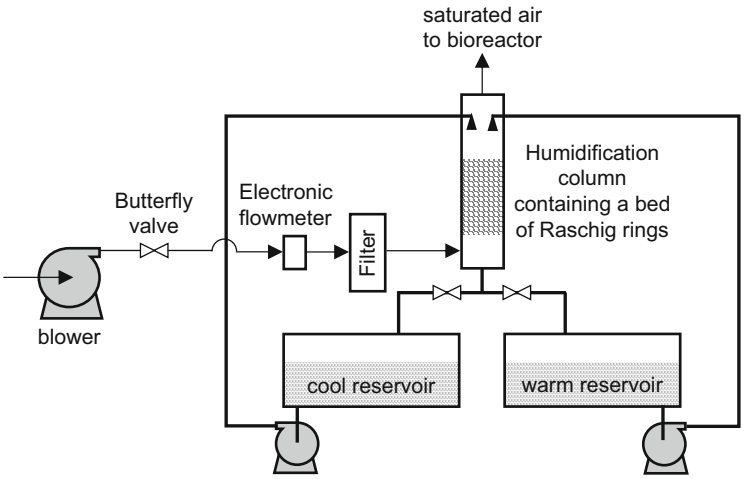


Fig. 4 Air preparation system for the pilot bioreactor. The humidification column can be fed from either the cool reservoir or the warm reservoir. When one reservoir is being used, the pump of the other reservoir is turned off, and the return valve of the other reservoir is closed. The temperatures of the water in the reservoirs are maintained using heating coils controlled by thermostats

would give a nominal superficial velocity (volumetric flow rate divided by the total cross-sectional area of the bed) of:

$$150\,\frac{\mathrm{m}^3}{\mathrm{h}}\left[\frac{10^6\ \mathrm{cm}^3}{1\ \mathrm{m}^3}\right]\left[\frac{1\ \mathrm{h}}{3{,}600\ \mathrm{s}}\right]/(60\ \mathrm{cm}\times 70\ \mathrm{cm})\approx 10\ \mathrm{cm\ s}^{-1}$$

The air was passed through a HEPA (high-efficiency particulate air) filter; however, no attempt was made to maintain the air line after the filter sterile, such that the main role of the HEPA filter was to remove dust from the inlet air.

Theoretically, it would be possible to supply air of any desired combination of temperature and humidity. This could be done by using electrical resistances to heat the air and injecting steam into it or, alternatively, by mixing dry and saturated air at the desired temperature [3]. However, these strategies are not simple to implement, with both requiring equipment for measurement and control.

In the end, we decided to use a much simpler design for the air preparation system, namely, a system with a humidification column, with this column being supplied from one of two water reservoirs (Fig. 4), each of which has a volume of 1,200 L. One reservoir, the "warm reservoir," was maintained at the optimal temperature for growth, and the other reservoir, the "cool reservoir," was maintained at a temperature several degrees lower. The humidification column contained a dispersion nozzle that showered water over a 70-cm-high bed of Raschig rings. The water was then returned to the reservoir from which it came. The bed of Raschig rings was oversized: it was designed by using a heat and mass balance model that suggested that saturation of the air would be achieved with a 35-cm-high bed.

The idea was that, at the beginning of the fermentation, it would be necessary to warm the bed to the optimal temperature for growth but that, once growth had started, it would be necessary to pass cooler air through the bed, approximately 5°C below the optimal temperature for growth, following the strategy suggested by Mitchell et al. [3]. Since the optimal temperatures for growth of the fungi that we were using (strains of *Aspergillus* and *Rhizopus*) were over 30°C and the room temperature typically did not exceed 25°C, it was not necessary to refrigerate the cool reservoir. In fact, during winter, it was typically necessary to heat the cool reservoir to around 25°C.

The disadvantage of our air preparation system is that we are limited to using saturated air at one of two temperatures and, therefore, we cannot promote evaporation by using partially saturated air. However, we considered that it is not particularly desirable to use unsaturated air in a packed-bed bioreactor, especially if the aim is to minimize the number of agitation events. Supplying unsaturated air to a packed-bed reactor can lead to the bed drying out significantly.

2.6 Monitoring Equipment

Thermocouples are placed at the air inlet and air outlet. Originally, combined temperature and relative humidity sensors were placed at these positions; however, they were not able to cope with the saturated air and lost their function. Four

thermocouple sleeves cross the bed horizontally, at different vertical and horizontal positions (Fig. 2d). Each sleeve contains four thermocouples. A piezoelectric differential pressure sensor is connected at the air inlet and air outlet. The readings of these sensors are logged every 18 s by LabVIEW. In some fermentations, we measured the O_2 and CO_2 concentrations in the outlet air, using an equipment that combines an electrochemical O_2 sensor with an infrared CO_2 sensor, with these readings also being logged by LabVIEW.

There is also an electronic flowmeter in the inlet air line. It measures the pressure drop across an orifice plate, but the reading is not logged by LabVIEW.

Although data is logged on the computer, due to cost considerations, the bioreactor does not have any computer-controlled systems. The temperatures in the water reservoirs for the humidification column are controlled automatically by thermostats that control electrical resistances. Each reservoir works at a fixed setpoint; the setpoint temperatures of the reservoirs are not manipulated during the process. The control of other aspects of bioreactor operation is operator-dependent. Although it would be possible to use an automated system to measure bed temperatures and control the pumps feeding the humidification column and the valves in the pipes returning the water from the humidification column to the reservoirs, in our case, this switching is done manually by the operator (Fig. 4). Likewise, if it is decided that it is necessary to rotate the bioreactor to mix the bed, the operator must disconnect the inlet and outlet air hoses and the cables attached to the thermocouples and then turn on the electric motor manually.

A perforated tube was placed in the headspace of the bioreactor, with the intention of allowing water to be sprayed onto the bioreactor surface when the bioreactor was in the upright position during a mixing event. The idea was that the water would be added in several aliquots, with rotation of the bioreactor between additions. However, this tube did not give a sufficiently uniform water distribution. Water addition was therefore done by opening the lid of the bioreactor and using a spray bottle to mist water onto the top of the bed. Once again, the water was added in several aliquots, with rotation of the bioreactor between additions.

2.7 Sampling from the Bed

Removing samples from packed-bed SSF bioreactors is problematic. The removal of samples from within the bed can lead to the formation of preferential flow paths. We were aware of this but were also interested in monitoring the development of axial gradients of moisture content and product formation in the bed. We therefore developed a sampler consisting of a screw auger inside a sleeve (Fig. 5a). During a completely static fermentation, we used this sampler to remove a "core" from the bed. We then took fresh substrate that had been prepared as for a fermentation, but which had not been inoculated, and pressed it into the vertical hole that had been left by the removal of the sample. However, when we opened the bioreactor later during the fermentation to take another sample, the fresh substrate that we had pressed into

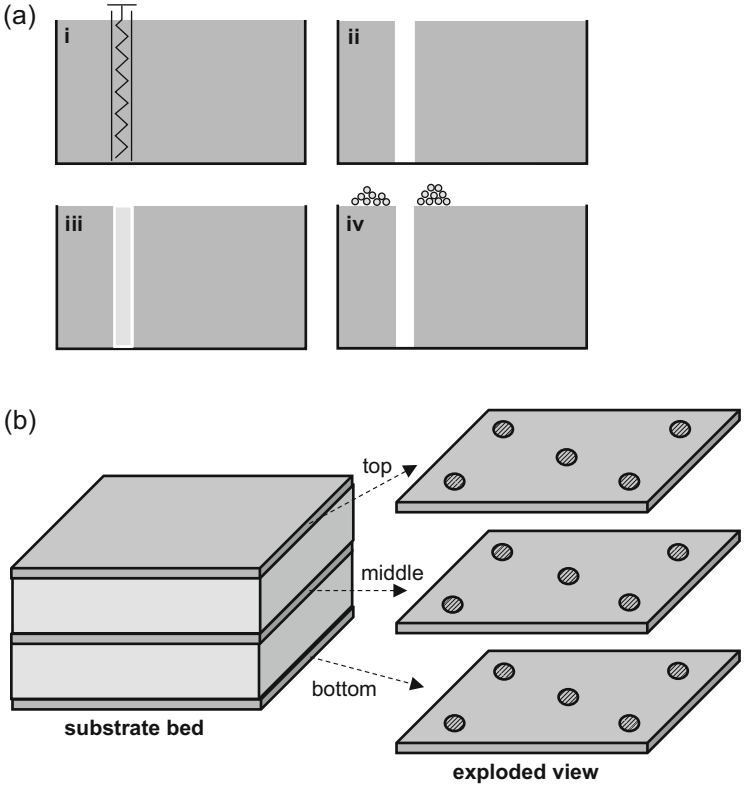


Fig. 5 Sampling from the bed of the pilot bioreactor. (**a**) Attempts to remove a core from the bed during the fermentation. Key: (i) Use of a screw auger to remove a core from the bed; (ii) vertical hole in the bed after removal of the core; (iii) vertical hole packed with uninoculated substrate; (iv) by the next sampling time, the uninoculated substrate had been blown out of the hole by the forced aeration. (**b**) Mapping of the bed at the end of the fermentation. Three horizontal planes were sampled, at the top, middle, and bottom of the bed. Five samples were removed from each plane, at the locations marked ⌀

the hole was on top of the bed, and the hole was fully open, with the air flowing preferentially through this hole, rather than through the bed. After this, we decided not to try to remove samples from within the bed during unmixed fermentations. In unmixed fermentations, we limited ourselves to removing samples from the top of the bed. Of course, in a fermentation with intermittent agitation, it is possible to remove samples from within the bed without disturbing the bed, if this is done immediately prior to the mixing event.

At the end of the fermentation, it is possible to sample the bed destructively in order to "map" gradients of moisture content and product levels (e.g., enzyme activities) in three dimensions. Layers of substrate can be carefully removed, with samples being removed from as many horizontal and vertical positions as is desired (Fig. 5b).

In some fermentations, we had an interest in following the evolution of gradients in a static bed during the fermentation. For this, it was necessary to undertake replicate fermentations and sample each one destructively at a different fermentation time. In other words, in order to characterize the spatial moisture content and enzyme activity gradients within the bed, we undertook three different but identical fermentations, stopping each one at a different time and sampling it destructively [11]. Two such experiments were done. For a completely static bed, the three fermentations were stopped at 12, 20, and 26 h. For a bed mixed three times (at 8, 10, and 12 h), the three fermentations were stopped at 15, 20, and 26 h.

The effect of mixing on spatial homogeneity within the bed is discussed in Sect. 4.1. However, these experiments also gave an insight into the reproducibility between identical fermentations carried out at different times. We plotted the temporal profiles for the enzyme activity of samples removed from the top of the bed. For the bed mixed three times, the temporal profiles for the fermentations stopped at 15, 20, and 26 h were quite similar to one another. For the completely static bed, there was significantly more variation among the profiles for the fermentations stopped at 12, 20, and 26 h [11].

3 Preparation of Substrate, Bioreactor, and Inoculum for a Pilot-Scale Fermentation

In order to undertake a fermentation, it is necessary to prepare both the substrate and an inoculum in sufficient quantities and to prepare the bioreactor.

3.1 Preparation of the Substrate and the Bioreactor

The rectangular substrate chamber of the bioreactor has a base of 60 cm by 70 cm and a height of 50 cm (Fig. 2b). When this chamber is full, the bed occupies 210 L. Of course, the mass of fresh substrate that can be held within this volume varies, depending on several factors: (1) the density of the substrate particles themselves; (2) the size and shape of the substrate particles, which affect how they pack; (3) the loading procedure, especially whether or not any pressure is applied to the substrate bed during packing; (4) whether or not the substrate bed is mixed, as this affects the packing of the particles; and (5) the water content used [12].

We used masses of up to 100 kg of dry substrate (50 kg soybeans plus 50 kg wheat bran) plus 40 kg water in fermentations undertaken with *Aspergillus oryzae* [13]. In fermentations undertaken to produce pectinases, we used smaller masses. For example, for the production of pectinases by *Aspergillus niger*, we used 27 kg of wheat bran and 3 kg of sugarcane bagasse plus around 50 kg of water, in a 40-cm-high bed [14]. For the production of pectinases by *Aspergillus oryzae*, we used

7.3 kg of sugarcane bagasse, 7.7 kg of citrus pulp, and about 50 kg of water, again in a 40-cm-high bed [15]. For the production of lipases by *Rhizopus microsporus*, we used 7.5 kg of wheat bran and 7.5 kg of sugarcane bagasse (dry mass) and 25 kg of water, which, once again, gave an initial bed height of 40 cm [16]. Obtaining and processing such masses of substrate raise challenges that are usually not faced when performing laboratory-scale SSF processes, which typically require the preparation less than 2 kg of substrate.

Often, one would like to run several fermentations with the same lot of substrate, in an attempt to avoid variation in bioreactor performance due to lot-to-lot variations of substrate properties. In order to do this for fermentations in a pilot bioreactor, it might be necessary to obtain a hundred or even several hundred kilograms of substrate. Even if the substrate is a residue and available free of charge, this is still problematic. A company will typically supply the residue free of charge at the location where it is generated and in the particular physicochemical state in which it is generated. It is then necessary to transport the substrate to the laboratory, incurring transportation costs. The substrate may be moist or wet when generated and, therefore, prone to natural fermentations that consume nutrients and generate inhibitory metabolites. It is, therefore, essential to obtain the residue as soon as possible after it has been generated, transport it immediately, and dry it without delay. For many research laboratories, suitable equipment for drying hundreds of kilograms of moist solids may not be easily available.

It may be necessary to process the substrate, for example, by chopping or grinding. For laboratory-scale fermentations, it is common to sieve the substrate by hand to ensure a uniform particle size (or range of particle sizes). To sieve many kilograms of substrate for a pilot fermentation, a vibratory sieve with sufficient capacity would be necessary. However, even if such equipment is available, one needs to consider carefully whether sieving is appropriate; if the substrate is sieved, a significant part of it may be wasted. The necessity of any other operations should also be carefully considered. For example, when we use sugarcane bagasse as a bed porosity modifier at laboratory scale, we normally wash the bagasse to remove residual sugars, with the intention of preventing these sugars from causing catabolic repression of enzyme production. However, this generates a large amount of residual wash water. The necessity of washing must be evaluated. In other words, the gain in fermentation performance due to removal of the residual sugars needs to be balanced against the costs of treating the wash water.

Also, at laboratory scale, it is common to add nutrients to the solid substrate. For some substrates, it is necessary to add a nitrogen source. Various inorganic salts may also be added because they lead to better growth and product formation. However, the real necessity for such additives needs to be evaluated. Pitol et al. [16] did this for lipase production by *R. microsporus* in SSF. At laboratory scale, we had been using washed sugarcane bagasse, impregnated with a medium containing soybean oil, urea, lactose, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, and oligoelements (ethylenediaminetetraacetic acid, $MnCl_2 \cdot 4H_2O$, $CoSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $CuCl_2 \cdot 2H_2O$, and $ZnSO_4 \cdot 7H_2O$) [17]. Before undertaking pilot-scale studies, we investigated the possibility of using a simpler medium. A medium composed of sugarcane bagasse, wheat bran, and urea

gave a price, per kg, that was a third of that of the medium that we had been using. Lipase production was not adversely affected. Pitol et al. [16] also reduced the costs of preparing the sugarcane bagasse, by eliminating the steps of washing, drying, and sieving of the sugarcane bagasse that we had been using previously [17].

Of course, the substrate needs to be autoclaved before the process, and the bioreactor also needs to be sterilized. Since our bioreactor was not designed as a pressure vessel, the substrate is autoclaved in a 300 L autoclave. Between fermentations, the bioreactor is cleaned by washing with neutral detergent. This is rinsed off, and then the bioreactor surfaces are sprayed with a food-grade sanitizing product containing peracetic acid and hydrogen peroxide. This is left for 30 min and then rinsed off.

3.2 Preparation of Sufficient Inoculum and Inoculation

The production of inoculum for laboratory-scale SSF processes is typically easy. However, when one needs to inoculate many kilograms of substrate, it can be a challenge.

We inoculated our pilot fermentations with spore suspensions. This is the most convenient form of inoculum for most filamentous fungi, although spore inocula result in long lag times at the beginning of the fermentation, since the spores take several hours to germinate.

The strains of *Aspergillus* that we used, *A. niger* and *A. oryzae*, sporulate easily. We prepared the inoculum by SSF in Erlenmeyer flasks. In order to inoculate 30 kg (dry mass) of substrate, Pitol et al. [14] and Finkler et al. [11] prepared a spore inoculum of *A. niger* using 40 250-mL Erlenmeyer flasks, each containing 10 g (dry mass) of a mixture of 30% sugarcane bagasse and 70% wheat bran (30:70 by mass). This mixture was autoclaved and then wetted with an ammonium sulfate solution to obtain a moisture content of 50% (m/m, wet basis). After inoculation, at 1×10^7 spores per gram of dry solid substrate, the flasks were incubated for 7 days, and then the spores were suspended in sterile distilled water. This process resulted in a spore concentration of 2×10^7 spores mL^{-1}. Likewise, in order to inoculate 15 kg (dry mass) of substrate, Biz et al. [15] prepared a spore inoculum of *A. oryzae* using 20 500-mL Erlenmeyer flasks, each of which contained 20 g of a mixture of citrus pulp and sugarcane bagasse (52:48 by mass). This mixture was autoclaved and then wetted with an ammonium sulfate solution to obtain a moisture content of 60% (w/w). After inoculation, at 4×10^7 spores per gram of dry solid substrate, the flasks were incubated for 6 days, and then the spores were suspended in sterile distilled water. The suspension was filtered through sterile gauze to remove residual substrate. This process resulted in 6 L of spore suspension, with 1×10^8 spores per mL.

When we tried a similar strategy with *R. microsporus*, producing spores in Erlenmeyer flasks with the fermentation substrate (a 50:50 mixture of wheat bran and sugarcane bagasse), the fungus did not sporulate well. We therefore had to develop a process to induce better sporulation [16]. The final process involved six

stainless-steel trays (each with a base of 19 cm × 26 cm and a height of 3.5 cm), each containing 183 g (dry mass) of a mixture of 86% parboiled rice and 14% rice husk. The wetting solution, prepared by boiling diced potatoes (300 g per liter of water), was added to give a low initial water content of 37.5% (w/w, wet basis), which favors sporulation of this fungus. After autoclaving, a spore suspension was added (1.7×10^7 spores per gram of dry solid substrate), and the inoculated substrate was placed in the trays, with a 30 mm bed depth. After 7 days of incubation at 30°C, a spore suspension was obtained by adding 5 mL of sterile Tween 80 solution (0.01% w/v) per gram of dry substrate. The spore suspension was filtered through sterile gauze to remove residual substrate.

Spreading the inoculum uniformly is essential to ensure that all particles are inoculated, avoiding extra lag time due to the need for the fungus to colonize uninoculated particles. However, adding the inoculum uniformly to a large mass of solids can be challenging. One needs to be careful with the addition of wet inoculum. If the liquid is added to the top of a mass of substrate and is given time to absorb into the substrate before the bed is mixed, then the transfer of spores from these top particles to the other particles during mixing may not be particularly effective. Ideally, the inoculum should be applied as a fine spray onto the substrate as it is being mixed. Potentially, this could be done within the bioreactor itself. In our pilot bioreactor, one possibility would be to add the inoculum in various aliquots. There would be repeated cycles in which a small volume would be sprayed onto the top of the bed (either through a well-designed nozzle or manually with a spray bottle) and then the bed would be mixed thoroughly. The addition of the inoculum could also be done in a dedicated mixing vessel. In this case, the mixer would turn the substrate bed continuously, with the addition of the inoculum as a continuous spray. In our case, we added the inoculum manually. The substrate was autoclaved in several bags, each containing a few kilograms of substrate. Appropriate aliquots of the spore suspension were added directly to each bag, and then the contents were immediately mixed, thoroughly, within the bag. The contents were then added to the bioreactor. Once the bioreactor was fully loaded, it was rotated for several minutes to mix the contents thoroughly. A manual procedure like this is feasible at pilot scale but would soon become impractical as the scale is increased further, making it essential to develop an automated system.

4 Production of Enzymes in the Pilot Bioreactor

This section describes our studies of the production of pectinases and lipases in the pilot bioreactor. The focus is not on comparing our results with other reports of the production of pectinases and lipases in SSF (for this, the reader should refer to the original articles). Rather, we focus on operational issues, such as the formation of gradients of temperature and moisture content within the bed, and how this is affected by agitating the bed and by using an inert material, such as sugarcane bagasse, to ensure a high porosity.

In this section, various different pectinase activities and lipase activities are given. The definition of the units (U) of activity can be found in the original papers. These definitions are not of key importance in the current context, but it is important to note that the activity units can only be used to compare the different fermentations undertaken by our group. Since other authors have used many different conditions for their activity assays, especially in the area of pectinases, it is not possible to compare our activity units with activity units given in the literature [18].

4.1 Production of Pectinases on Wheat Bran

Our first studies involved the production of pectinases by *A. niger* cultivated on wheat bran, in fermentations lasting up to 26 h [11, 14]. In these fermentations, the air entered the bioreactor at 150 m^3 h^{-1} (nominal superficial velocity of 0.1 m s^{-1}) and at a temperature around 30°C.

We initially undertook fermentations without any attempt at control: we did not agitate the bed, and we did not change the temperature of the water being fed to the humidification column [14]. In the first fermentation, the substrate bed consisted entirely of wheat bran (20 kg dry mass). Around 17 h of fermentation, the bed shrank away from the bioreactor walls. With the preferential flow of air through the gaps next to the wall, the bed was no longer properly aerated, and temperatures as high as 37°C were recorded in the bed [14]. At this time, since the air was passing around the bed, not through it, the air temperature in the headspace above the bed was lower, ranging between 30 and 32°C. This "inversion of temperatures," namely, the bed temperature being higher than the air temperature in the headspace, is a clear sign of preferential flow and could be used in an automated system to trigger an alert, informing the operator of the need to take corrective action. When the fermentation was ended at 24 h, large compact agglomerates were encountered within the bed during the unloading of the bioreactor.

In the second fermentation, we attempted to avoid the problem of bed shrinkage by replacing 10% of the wheat bran with sugarcane bagasse (i.e., dry masses of 18 kg wheat bran and 2 kg sugarcane bagasse). The addition of sugarcane bagasse increased the porosity of the bed: whereas the bed height at the start of the first fermentation was 23 cm, it was 27 cm at the start of the second fermentation. The fermentation was successful, in that the bed did not shrink away from the wall and the bed temperature was well controlled, not exceeding 31°C. Based on the poor performance of the first fermentation, we decided to "map" the pectinase activities in the bed at the end of this second fermentation, at 26 h (see Fig. 5b). Since the bed conditions were well controlled, the pectinase activity did not vary significantly: the values ranged from 17 to 20 U g^{-1} [14].

Encouraged by the success of the addition of sugarcane bagasse, we decided to use the same substrate mixture, but to increase the overall mass of the bed by 50%: 27 kg of wheat bran and 3 kg of sugarcane bagasse (dry masses), giving an overall bed height of 40 cm. Our thinking was that a packed-bed bioreactor should have as

high a bed height as possible: for a given mass of substrate, a higher bed height would give a smaller bioreactor footprint. However, with the increased bed height, compaction problems returned. At 16 h, the bed shrank away from the bioreactor wall, and temperatures as high as 43°C were measured in the upper regions of the bed. With this high temperature, the pectinase activity of samples removed from the top of the bed decreased, from a value of 23 U g^{-1} at 14 h to 13 U g^{-1} at 20 h. When the bed was mapped at 26 h, the pectinase activities varied significantly: from 16 to 28 U g^{-1} at the bottom of the bed, from 11 to 19 U g^{-1} in the middle of the bed, and from 13 to 20 U g^{-1} at the top of the bed [14].

In a following study, we maintained the same substrate, microorganism, and fermentation conditions but investigated whether agitation of the bed could prevent the bed from pulling away from walls, thereby promoting uniformity of pectinase levels [11].

Four mixing regimes were compared: (1) a completely static bed, (2) one mixing event (at 10 h), (3) three mixing events (at 8, 10, and 12 h), and (4) five mixing events (at 8, 10, 12, 14, and 16 h). Agitation of the bed enabled the bed temperature to be controlled well: over a period of at least 3 h after a mixing event, the bed temperature did not exceed 35°C [11], whereas in the absence of mixing, bed temperatures over 40°C had been measured [14]. However, if the bed was left static for longer periods (about 8 h) after an agitation event, evidence of the formation of preferential flow paths appeared (i.e., bed temperatures that were higher than the headspace temperature). Mixing promoted uniformity of temperatures across the bed: in the mixed fermentations, the thermocouples within the same horizontal sleeves gave readings that typically varied less than 2 or 3°C from one another. On the other hand, after 15 h of fermentation in a static bed, thermocouples within the same horizontal sleeve sometimes gave readings that were 5–17°C different [11].

As a result of the poor temperature control in the static fermentations, the pectinase activities of samples removed from the top of the bed varied by up to 10 U g^{-1} toward the end of the fermentation (from 13 to 23 U g^{-1} after 14 h). On the contrary, the pectinase activities of samples removed from the top of the bed for fermentations with three or more mixing events were very close to each other up to 16 h. After 16 h, they varied more but still by less than 5 U g^{-1} for samples removed at the same fermentation time [11].

When the bed was mapped at the end of the 26-h fermentation, the fermentation with five mixing events had the most uniform distribution of pectinase activities, with the average pectinase activities at the bottom, middle, and top of the bed all falling in the range of 22–23 U g^{-1}. This fermentation also gave the best uniformity within horizontal planes, with sample standard errors (calculated for the five samples collected at the same bed height) being below 2 U g^{-1}. However, the use of five mixing events was slightly deleterious. The highest overall pectinase production at 26 h was obtained in the fermentation with three mixing events, although the activities were less uniform within the bed. In this case, the average pectinase activities ranged from 29 U g^{-1} at the bottom of the bed to 22 U g^{-1} at the top of the bed, and the sample standard errors calculated for five samples removed from the same horizontal plane ranged from 2 to 4 U g^{-1} [11].

An important conclusion from the study of the effect of mixing is that, if the bed is heterogenous, then removing samples only from the top of the bed (in order not to disturb the bed structure) can lead to erroneous decisions about the best agitation strategy and the best harvesting time. Based on samples removed from the top of the bed, it would appear that the static fermentation might be best, since an activity of 23 U g^{-1} was obtained at 14 h. However, due to the relatively poor uniformity within the static bed, there is no guarantee that this value applies to the whole bed. In fact, based on mapping of the whole bed at 20 h, the fermentation with three mixing events had an average pectinase activity of 22 U g^{-1}, which was significantly higher than the average pectinase activity of 18 U g^{-1} for the static fermentation [11].

4.2 *Production of Pectinases and Lipases in Beds with High Sugarcane Bagasse Contents*

We also did fermentations with high contents of sugarcane bagasse: the production of pectinases by *A. oryzae* [15] and the production of lipases by *R. microsporus* [16]. In both cases, the initial bed height was 40 cm. In the case of the production of pectinases by *A. oryzae*, we used 7.3 kg of sugarcane bagasse and 7.7 kg of citrus pulp (dry masses), with about 50 kg of water [15]. For the production of lipases by *R. microsporus*, we used 7.5 kg of wheat bran and 7.5 kg of sugarcane bagasse (dry masses), with about 28 kg of water [16].

With the high content of sugarcane bagasse in these two systems, we managed to avoid the problems that Pitol et al. [14] encountered, namely, the formation of compact agglomerates of substrate and bed shrinkage. As a consequence, we avoided the formation of preferential flow paths and overheating of the bed. In both cases, the temperatures throughout the substrate bed remained very close to the inlet air temperature [15, 16]. It was, therefore, not necessary to use the cooler reservoir to provide cool air to the bed, a strategy that Pitol et al. [14] had tried during a static fermentation, unsuccessfully. The good temperature control of the bed had an added benefit: saturated air was supplied to the bed and, in the absence of axial temperature gradients, the water carrying capacity of the air did not increase as it flowed through the bed. Consequently, the moisture content of the bed remained close to the initial water content throughout the fermentation. In various fermentations reported by Pitol et al. [14] and Finkler et al. [11], the moisture content decreased by over 10 percentage points during the fermentation. Of course, the advantage of using inert materials that do not shrink during the fermentation in packed-bed bioreactors has been known for many years [19].

In the production of pectinases by *A. oryzae*, the sugarcane bagasse was added simply as a bed porosity modifier; it was not impregnated with nutrients. This inert material represented half of the mass of the substrate bed, and the bulk density of the bed was only about 90 kg of dry substrate per m^3 [15], a half of the value of 180 kg of dry substrate per m^3 for the wheat bran medium used by Pitol et al. [14]. Potentially,

the use of a medium with a much lower bulk density would lead to a much lower overall production of pectinases in the bioreactor. However, the pectinase activity per gram of dry substrate obtained by Biz et al. [15], 37 U g^{-1}, was much higher than the best value of 20 U g^{-1} obtained by Pitol et al. [14]. As a result, Biz et al. [15] obtained a total of 555 $\times$ 10^3 U in the bioreactor, which is more than 90% of the value of 600 $\times$ 10^3 U obtained by Pitol et al. [14].

5 The Pilot Bioreactor and Mathematical Models in Scale-Up Studies

Studies within a pilot bioreactor are not an end in themselves. Rather, these studies should provide a basis that guides the design and operation of a full-scale, commercial bioreactor that contains hundreds of kilograms, or even several tonnes, of substrate.

Our pilot bioreactor allows for the use of substrate beds of heights up to 50 cm, a bed height that has been used in some large-scale packed beds [20]. If the fermentation in the pilot bioreactor is successful, then one possible scale-up strategy is to maintain the same bed height and simply increase the width of the bioreactor. In this case, phenomena that depend on the bed height, such as axial temperature gradients and pressure drops across the bed, can be studied at pilot scale using the same bed height that will be used at commercial scale.

If the same bed height is maintained, one possibility would be to use the same design as we used and simply increase the length of the central axis of the cylinder. In this case, a larger-scale bioreactor would be intermittently mixed using the same tumbling action that we used for mixing in our pilot bioreactor. Of course, other designs could be used, such as the circular beds used in the production of soy sauce *koji* [20]. Such designs use mechanical agitators to mix the bed (Fig. 3).

However, it is also useful to consider using bed heights higher than 50 cm at commercial scale, since for the same amount of substrate, a higher bed height will give a smaller bioreactor footprint. The possibility of using higher bed heights than those studied at pilot scale can be investigated using mathematical models of heat and mass transfer. These models predict axial temperature and moisture gradients, identifying the potential of high bed temperatures or low water activities occurring in particular regions of the bed.

Various models have been proposed that can be used as tools for guiding the scale-up of packed-bed bioreactors. The first models assumed that, as the air passes through the bed, it remains in thermal and moisture equilibrium with the solids. Effectively, this is equivalent to the assumption that, as the air heats up due to the removal of metabolic heat from the solids, water evaporates from the solids to the air to maintain the air saturated. When our research group first proposed scale-up strategies for packed-bed SSF bioreactors, for both the traditional design and the Zymotis design, we used models based on this assumption [6, 10]. However, Weber

et al. [21] showed that the air and solids are not necessarily in equilibrium in a packed-bed bioreactor, especially during the periods of most rapid microbial growth: in their studies, the relative humidity of the outlet air fell to values below 90%. When we developed a model to be used as a tool in guiding the design and operation of our forcefully aerated, intermittently agitated, pilot bioreactor, we recognized that the solid phase and gas phase are not necessarily in equilibrium [22]. As such, we called it a "two-phase model."

Our "two-phase model" describes heat transfer only along the direction of the airflow [22]. Casciatori et al. [23] extended the two-phase modeling approach by including the radial coordinate. In other words, in addition to describing heat and mass transfer by conduction, convection, and evaporation along the central axis of the bioreactor (i.e., in the axial direction, parallel to the airflow), they also described heat transfer by conduction from the central axis to the bioreactor wall (i.e., in the radial direction, normal to the airflow). However, although it may be appropriate to describe radial heat transfer in order to model the performance of a thin, water-jacketed, laboratory-scale packed bed, when the aim is to use the model to guide the scale-up of a traditional packed-bed bioreactor, there is no advantage in describing heat transfer in the radial direction. At large-scale, traditional packed beds are typically several meters wide, and conductive heat transfer radially to the bioreactor wall influences only a few centimeters near the bioreactor wall, making a negligible contribution to bed cooling. In fact, this consideration means that when one is using a thin packed bed in studies aimed at guiding the design and operation of a traditional large-scale packed-bed bioreactor, one should insulate the sides of the thin packed bed rather than using a water jacket. On the other hand, describing heat transfer normal to the airflow is useful in guiding the scale-up of Zymotis-type packed-bed SSF bioreactors. A model of this type of bioreactor was developed by Mitchell and von Meien [6] and used to show that the internal heat transfer plates need to be less than 10 cm apart in order for the bed temperature to be well controlled [7]. This model assumed thermal and moisture equilibrium between the air and solid phases. Such a model could benefit from incorporating the two-phase approach.

The two-phase model of von Meien and Mitchell [22] was a useful tool for investigating control strategies [24]. Two control strategies were investigated, PID (proportional-integral-derivative) and DMC (dynamic matrix control). The aim was to maintain the average bed temperature as close as possible to the optimal temperature for growth. Two aeration strategies were compared: (1) maintaining the air essentially saturated (relative humidity of 99%) while varying its temperature and (2) maintaining the air at the optimal temperature for growth while varying its humidity. The former strategy was predicted to give better control of the average bed temperature and, therefore, a better predicted growth profile (representing the average biomass content of the bed). DMC was predicted to give better growth than PID. A key conclusion of the work was that the availability of the two-phase model allowed a relatively inexpensive investigation of different control strategies for the pilot bioreactor. The costs in terms of both material and time would be much greater if it were necessary to undertake this work solely experimentally. Of course, it is essential to confirm that the most promising control strategy identified in the simulation work does indeed lead to a high level of

performance of the bioreactor, but this confirmation can be done with a relatively small number of experiments.

Computational fluid dynamics (CFD) can be used to model SSF processes in bioreactors. It has been applied to describe the initial phase of heating of the substrate bed during the lag phase in our pilot bioreactor [25] and the initial stages of a fermentation itself [26]. Given the long simulation times, which can be of the order of several weeks, CFD has most to offer in situations that cannot easily be modeled with classical continuous differential equations. Continuous differential equations (either ordinary or partial) can be used to model simple geometries, such as cylinders, and smooth gradients in space (in axial or radial directions). CFD is useful for complicated geometries, of the bioreactor itself, or of the air supply. It is also useful when the bed contains obstacles and when the bed has irregular properties, which might occur through nonuniform packing or the opening up of preferential flow paths (in the form of cracks or gaps). Since CFD programs were not originally designed for describing fermentations, it is necessary to use user-defined functions to simulate many of the phenomena that occur during SSF, such as microbial growth and particle shrinkage.

Mathematical models will only represent fermentations well if the correlations and values used in them are correct, so it is important to characterize the properties of the beds used in packed-bed bioreactors. The two-phase model of von Meien and Mitchell [22] relies heavily on data obtained in studies of the drying of grains that are different from the substrates that we have used for the production of pectinases and lipases. Recently, hygroscopic properties have been published for substrates that we used in our fermentations, including properties of orange pulp [27], sugarcane bagasse, and wheat bran [28]. It is important to note that studies of the hygroscopic properties of substrates used in SSF processes usually focus on characterizing the original substrate, as prepared for a fermentation. However, the isotherms obtained for the original substrate might not apply during the fermentation, since the biomass can have hygroscopic properties that are significantly different from those of the substrate [29]. Data have also been published for key structural properties of beds packed with sugarcane bagasse, wheat bran, and orange pulp and peel and with mixtures of these substrates [12]. These properties include the bulk packing density and the bed porosity; they were characterized as functions of the bed moisture content and the packing technique [12].

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It Is the Mix that Matters: Substrate-Specific Enzyme Production from Filamentous Fungi and Bacteria Through Solid-State Fermentation

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Contents

Abstract Fungi have a diverse spectrum of extracellular enzymes. In nature, extracellular enzymes primarily serve to procure nutrients for the survival and growth of the fungi. Complex polymers such as lignocellulose and starch as well as proteins and fats are broken down into their basic building blocks by extracellular enzymes such as amylases, proteases, lipases, xylanases, laccases, and many more.

The abilities of these enzymes are made use of in diverse areas of industry, including food technology, textiles, and pharmaceuticals, and they have become indispensable for today's technology. Enzyme production is usually carried out using submerged fermentation (SmF). However, as part of the search for more sustainable uses of raw materials, solid-state fermentation (SSF) has become the focus of research.

The rate of enzyme formation depends on different factors, for example, microorganism, temperature, or oxygen supply. However, one of the most important factors in enzyme production is the choice of substrate, which varies depending on

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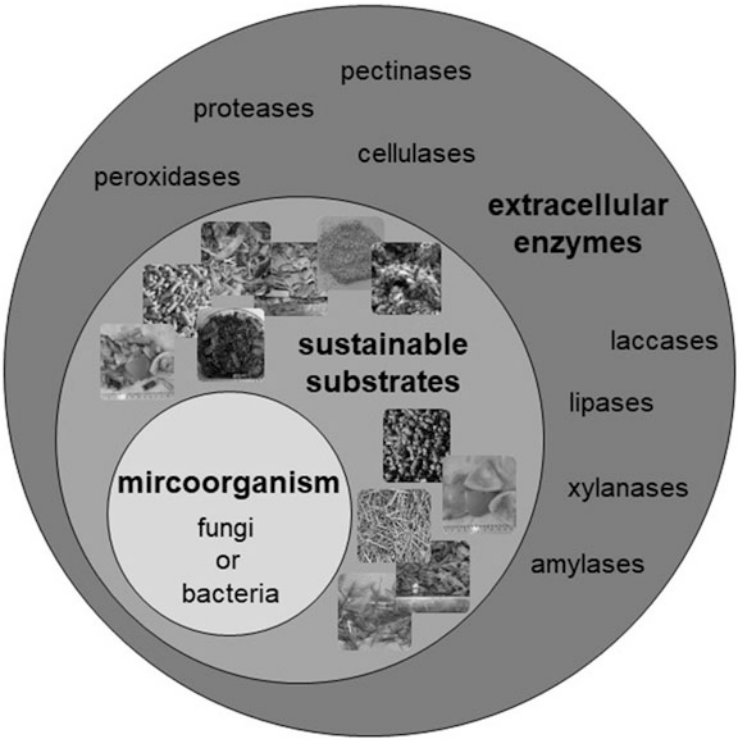
the desired target enzyme. Substrates with proven effectiveness include wheat bran and straw, but unusual agricultural residues such as forage cactus pears and orange peels have surprisingly positive effects on enzyme formation as well.

This review gives an overview of various technically relevant enzymes produced by filamentous fungi and suitable substrates for the production of the enzymes by SSF.

Graphical Abstract



Keywords Basidiomycetes, Extracellular enzymes, Filamentous fungi, Residues, Solid-state fermentation, Substrate-specific enzyme formation, Sustainability

1 Introduction

Nature fascinates us with its incredible diversity and wide array of possibilities. A variety of organisms has arisen through millions of years of evolution, generating metabolic pathways and resulting products which are highly interesting for industry.

This review focuses on extracellular enzymes, predominantly those formed by bacteria and fungi and used in different industries where the breakdown of complex macromolecules or the improvement of digestibility, solubility, or viscosity is desired. In nature, these enzymes are mainly used for substrate degradation and nutrient extraction. Organisms are able to form special enzyme cocktails for each specific substrate.

In order to obtain these specialized enzymes, it is sometimes necessary to simulate the natural habitat of the organisms. One possibility for the implementation of preferred terrestrial systems in industry is solid-state fermentation (SSF).

The term solid-state fermentation applies to all forms of fermentation involving solid particles in the absence of a free liquid phase. This means the organism is grown on a bed of solid particles, with the space between the particles consisting of a continuous gas phase and liquid being retained by the solid particles, which fulfil the following three important functions: carrier material, nutrient source, and moisture reservoir. In addition to classical solid-state fermentation, various mixed forms have also become established, such as solid-substrate fermentation. Solid-substrate fermentation is a form of liquid cultivation (submerged fermentation, SmF) used predominantly for industrial purposes, with the addition of solid particles as complex substrates.

In addition to the cultivation method, various parameters have a significant influence on enzyme formation. These include, among others, culture time, culture temperature, inoculation level, humidity, initial pH, aeration, agitation (mixing speed, mixing duration, and mixing rate), and particle size. However, the choice of substrate remains an important factor, as different enzymes are specifically induced by various complex solid substrates.

To provide an overview of the main enzymes produced by filamentous fungi, along with their relevant substrates, this review evaluates the substrate-specific formation properties of most studied extracellular enzymes. Various enzymes and enzyme groups relevant for industry are compared and analyzed along with their preferred substrate for fermentation, and results from our own work are presented.

2 Enzyme Production Through Solid-State Fermentation

Solid-state fermentation is ideal for cost-effective and substrate-optimized production of extracellular enzymes by filamentous fungi, whose implementation usually follows the same principle that has hardly changed in recent years.

First, the substrates were sterilized and inoculated. The incubation took place over several days to weeks depending on the organism and the target enzyme. Most

of the studies were still carried out in static shake flasks (usually 250 mL) with 5–10 g of substrate filling [1–10], but investigations on a larger scale were also conducted. In this case, the substrate bed either remained static (e.g., tray reactors) [11–17] or was occasionally circulated (e.g., drum reactors) [18]. Also, oxygen supply to the organisms used and heat dissipation were supported by partially enhanced ventilation [19, 20].

The extracellularly formed enzymes were finally obtained as a crude solution through enzyme extraction. To achieve this, the overgrown substrate was mixed with water or buffer (e.g., phosphate buffer or acetate buffer) [2, 3, 6, 9, 21–24]. The mixing was carried out on a laboratory scale by shaking or stirring, usually for 30 min to 2 h (time periods of 5 min to 4 h were also used), at 100–250 rpm [2, 9, 14, 15, 25–30]. Subsequently, the solid particles were separated by filtration or centrifugation. In some cases, additional centrifugation of the retentate was carried out [5, 10, 31–33]. A crude enzyme solution was recovered which could be used for further investigation or purification or used directly in the application. Additional purification steps included precipitation with ammonium sulfate and chromatographic purification steps [34, 35].

In recent years, a variety of different enzymes and their environmentally friendly production by solid-state fermentation have been investigated. The relevant enzymes can be roughly categorized into two major groups: (1) hydrolytic enzymes, such as amylases, pectinases, and proteases, and (2) lignocellulolytic enzymes, such as cellulases, xylanases, laccases, and various peroxidases. Other enzymes, such as phytases or tannases, have also been investigated by some research groups [36–39].

The formation rate and amount varied depending on the substrate and organism used. Preferred organisms were representatives of ascomycetes (e.g., *Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp.) and bacteria (e.g., *Bacillus* sp.), as well as basidiomycetes (e.g., *Pleurotus* sp., *Trametes* sp., *Phanerochaete* sp.) [1, 17, 21, 34, 40–46].

The substrates consisted mostly of residues from the agriculture, food, and forestry industries, such as crop residues, wood chips, fruit and vegetable peels, and other renewable raw materials. These substrates have high availability and are inexpensive, meaning production costs and negative environmental effects (due to, e.g., disposal) are reduced. In general, they can be divided into six categories: (1) sugary, such as sorghum and sugarcane bagasse, for the production of cellulases and xylanases [3, 4, 43]; (2) starchy, such as wheat bran, suitable for amylases [2, 47]; (3) lignocellulosic, such as fruit peels, straw, and sawdust, suitable for cellulases, xylanases, laccases, and peroxidases [5, 8, 46, 48]; (4) protein rich, such as soya residues, suitable for proteases [29, 49, 50]; (5) oily, such as olive pomace, for the production of lipases [19]; and (6) inert carriers, such as polyurethane foam [51, 52], used rarely or in combination with liquid fermentation.

In the following, some selected enzymes and their substrate-specific production are discussed in more detail.

3 Important Extracellular Enzymes

3.1 Selected Industrially Relevant Enzymes

In the following, various industrially relevant enzymes which can be produced by solid-state fermentation are presented. The main focus is on the selection of substrates used. It turned out that, in addition to the production strain used, the substrates have a considerable influence on the enzyme activity achieved. Most of all, the composition of substrates plays an important role. Thus, the individual components, as expected, have an inducing effect on the desired target enzymes.

3.1.1 Amylases

Amylases belong to the group of hydrolases and cleave the glycoside bonds of polysaccharides (primarily starch), which is why they are also classified as glycosidases. Amylases are subdivided into α-, β-, γ-, and iso-amylases and are produced by various organisms such as bacteria, fungi, plants, and animals. In particular, mainly the amylases of bacteria and fungi, especially α-amylase (EC 3.2.1.1), are used in industry and technology [53, 54].

Amylase makes up approximately 25% of the world enzyme market [55]. The main area of application is in the food industry. Here, amylases are used in the mashing process in breweries and for the pretreatment and modification of flour (for airier dough or a greater degree of browning) and starch, as well as in the production of glucose/maltose syrup and maltodextrin. They are also used in pharmacy or in dishwashing and laundry detergents for the removal of starchy stains and dirt, as well as in the production of animal feed and biofuels [53–55].

Amylases are produced both by submerged fermentation and by solid-state fermentation. Here, bacteria such as *Bacillus* sp. or fungi, especially ascomycetes, are preferably used [54]. However, alongside the introduction of new production strains, production has also been improved by the use of genetically modified or engineered strains [54]. Basidiomycetes were not in focus for the production of amylases.

Submerged fermentation usually uses expensive media. Costs can be reduced by the use of residues in solid-state fermentation. Wheat bran has been a popular and well-suited substrate for amylase production since the 1980s [47]. Recent studies have shown that other substrates are also suitable for the production of amylases (see Table 1). Substrates on which high enzyme yields were obtained were mostly those which still contained starch residues. For example, different types of bran (residues after the sieving of flour) have between 8% and 45% of starch (wheat 13–18%, oats 18–45%, rice 18–30%) [56]. However, in comparison to studies on wheat bran (63.25 U/g [57] and 14.5 U/min/mL [47]), investigations on laboratory scale (substrate use 5 g to 1.2 kg) showed that even substrates with small amounts of starch, such as wheat straw (6,900 U/gds (gram dry substrate)) [58], banana peels (42 U/mg) [59], mixtures of orange peels and cheese whey (220 U/mL) [1], and a mixture of soy

Table 1 Review of recent studies on amylase production on solid substrates (bold: best substrate) including achieved amylase activity, used production strain, and cultivation size [1, 2, 18, 25, 47, 50, 57–59, 61]

Substrate	Activity	Microorganism	Reactor		Ref.
Orange peels and cheese whey	220 U/mL	*Bacillus amyloliquefaciens*	Erlenmeyer flasks (250 mL)		[1]
Wheat straw, sugarcane bagasse, rice straw, and rice husk	6,900 U/gds	*Bacillus* sp. BBXS-2			[58]
Corn bran, barley bran, wheat bran	2.8 U/mg protein	*Rhizoctonia solani* AG-4 strain ZB-34	Erlenmeyer flasks (250 mL)	5 g	[2]
Sago hampas	1.055 ± 0.03 U/mL	*Aspergillus flavus* NSH9	Flasks	5 g	[25]
Raw banana peel, sugarcane bagasse, sawdust, potato peel, wheat bran, rice bran, cassava peel, orange peel	42 U/mg	*Microbacterium foliorum* GA2	Flasks	5 g	[59]
Wheat bran, corn straw, corn cob, rice peel, soy bran	63.25 U/g (6.32 U/mL)	*Gongronella butleri*	Erlenmeyer flasks (250 mL)	5 g	[57]
Maize pericarp, soybean meal, sunflower meal, maize bran, olive oil cake, and wheat bran	50.75 IU/g	*Bacillus* sp. TMF-1	Erlenmeyer flasks (150 mL)	5 g	[50]
Wheat bran, banana peels, rice bran and husk, gram husk, mineral salt solution	14.5 ± 0.1 U/mL/min	Bacterial co-culture: *Bacillus cereus* and *Bacillus thuringiensis*	Erlenmeyer flasks (250 mL)	10 g	[47]
Soy and bread waste mixture (90:10)	$39.9 \cdot 10^3$ U/gds	*Thermomyces* sp. ATCC-200065 and *Geobacillus* sp. ATCC-31198	Cylindrical reactors (4.5 L)	1.2 kg	[61]
Corn bran	20.68 ± 1.03 U/mL	*Bacillus amyloliquefaciens* NRRL B-645	Rotating drum bioreactor (7 L)	2.8–5.6 L	[18]

and bread wastes (90:10) (39,900 U/gds) [14] are suitable for amylase production. The substrates used have a low starch content (less than 3%) and a high non-starch carbohydrate content (e.g., hemicellulose, cellulose, pectin, etc.) of up to 60% (in banana peels) [60], which could also promote enzyme formation.

3.1.2 Proteases

Proteases cleave proteins through hydrolysis of peptide bonds. According to their catalytic mechanisms, they are subdivided into exoproteases (cleavage of fewer amino acids at the C- or N-terminus) and endoproteases (cleavage in the middle of the polypeptide chain). In addition, a differentiation is made of exoproteases into di-

and tri-peptidyl proteases and endoproteases into serine- (subtilisin), cysteine- (papain), aspartate- (pepsin), and metallo-proteases (thermolysin) [53].

The most important protease for industry is subtilisin, which is used in detergents and cleaning agents (global (2010), 2000 t/a [54]; Europe (2002), 900 t/a [53]). This enzyme is mainly produced in industry by bacteria of the species *Bacillus subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* [53, 54]. Further uses of proteases include the production of protein hydrolysates and baby food, and they also offer an environmentally friendly alternative in leather processing and the treatment of industrial and domestic wastewater [53].

Just as for amylases, industrial production of proteases usually involves submerged fermentation. However, the use of solid substrates is also possible. For this purpose, *Bacillus* species are still cultivated as the main producer in research [29, 49, 50, 58, 62].

Above all, protein-rich substrates of soy residue (protein content up to 50% [63]), wheat and rice bran (protein content 15–18% [56, 64]), or residues of animal origin such as cuttlefish (protein content 16–18% [65]) or cow hairs were shown to be suitable for the production of extracellular proteases (see Table 2). Thus, through fermentation in a 50 L adiabatic packet bed reactor with a loading of 20 kg soy fiber and 17.5 kg dehydrated sludge and cow hair, up to 33,374 U/gds of protease activity were achieved [66]. In addition to the use of *Bacillus* species, proteases with an activity of 463.5 U/mL could also be produced on a mix (3 kg) of cupuaçu exocarp and rice bran by the basidiomycete *Lentinus citrinus* (analysis of fruiting bodies) [24]. However, the yields are much lower than that achieved by the cultivation of *Bacillus* sp.

3.1.3 Lipases

Lipases, also referred to as triacylglycerol hydrolases, degrade fats to glycerol and free fatty acids or to mono- and diglycerides. They occur in fungi, bacteria, animals, and plants. Their most important properties include their broad substrate specificity, their high stability (including in organic solvents), as well as the conversion of many substrates with high stereo- and enantioselectivity [53, 54].

This means there are many applications for lipases, ranging from pharmaceuticals (e.g., ibuprofen production) to the production of enantiomerically pure fine chemicals and fragrances for use in the food industry (e.g., transesterification of wheat flour lipids or for the production of breast milk fat substitutes and cocoa butter substitutes). In addition, lipases are used as a detergent additive, in leather production (e.g., degreasing) and in the paper industry [53, 54].

Industrial production is carried out by fungi, preferably ascomycetes, such as *Aspergillus niger*, *Candida antarctica*, *Rhizomucor miehei*, and *Pichia* sp. [54]. The main issue in their production is usually the use of water-insoluble substrates, which can cause enzymes to accumulate in the interfaces and reduce yields [54].

In recent years there have been efforts in research to produce lipases using solid-state fermentation. Representatives of the ascomycetes continue to be preferred for

Table 2 Review of recent studies on protease production on solid substrates (bold: best substrate) including achieved protease activity, used production strain, and cultivation size [7, 24, 29, 49, 50, 58, 62, 66–68]

Substrate	Activity	Microorganism	Reactor		Ref.
Wheat straw, sugarcane bagasse, rice straw, and rice husk	12,200 U/gds	*Bacillus* sp. BBXS-2			[58]
Corn pericarp, soybean meal, sunflower meal, maize bran, maize pericarp, olive oil cake, wheat bran	50.5 IU/g	*Bacillus* sp. TMF-1	Erlenmeyer flasks (150 mL)	5 g	[50]
Wheat bran and rice bran, green gram husk, tapioca peel, banana peel	4,143 + 12.31 U/g	*Bacillus* sp. IND12	Erlenmeyer flasks	5 g	[62]
Cuttlefish waste and cow dung + C-source	1,218 ± 27 U/g	*Bacillus cereus* IND5	Erlenmeyer flasks (100 mL)	5 g	[29]
Wheat bran (WB) and rice flour (RF)	1193.77 U/g	*Brevibacterium luteolum* MTCC 5982	Erlenmeyer flasks (250 mL)	10 g WB 0.5 g RF	[67]
Cotton cake	728 U/mL	*Bacillus subtilis* K-1	Erlenmeyer flasks (500 mL)	10 g	[49]
Gram husk	714 U/mL				
Mustard cake	680 U/mL				
Soybean meal	653 U/mL				
Wheat bran, chicken feather maize bran, rice husk, cane bagasse, corn cob					
Soybean bran, wheat bran	115.6 U/mL	*Aspergillus oryzae* INCQS 40068 *and other ascomycetes*	Erlenmeyer flasks (250 mL)	10 g	[7]
Soy fiber mixed with wood chopsticks and wood chips	800 U/gds	*Thermus* sp. ATCC® 3,174	Bioreactor (500 mL)	115 g	[68]
	670 U/gds		Bioreactor (10 L)	2.3 kg	
Cupuaçu exocarp + rice bran, cupuaçu exocarp + litter	463.55 U/mL	*Lentinus citrinus (fruiting bodies)*	Polythene bags	3 kg	[24]
Agro-industrial wastes: Soy fiber, cow hair mixed with dehydrated sludge from wastewater treatment plant	33,374 U/gds (SF) 23,541 U/gds (HS)	n.n.	Adiabatic packet bed reactor (10 L)	3.8 kg SF + 2.8 kg HS	[66]
			Adiabatic packet bed reactor (50 L)	20 kg SF + 17.5 kg HS	

lipase production (see Table 3). Basidiomycetes play only a minor role as lipase producers.

As expected, lipid-rich substrates were used as preferred substrates, such as coconut meal, olive pomace, and palm kernel cake. These were used separately or in combination with nutrient-rich substrates, such as wheat bran. Wheat bran has a fat content of only 4–5%, but it contains a lot of carbohydrates (13–18% starch,

Table 3 Review of recent studies on lipase production on solid substrates (bold: best substrate) including achieved lipase activity, used production strain, and cultivation size [16, 19, 27, 33, 40, 69–74]

Substrate	Activity	Microorganism	Reactor		Ref.
Lipid-rich agro-wastes: **Coconut meal** with supplements, almond meal, brassica meal, sesame meal, rice, wheat bran	41.1 ± 0.4 U/mL/min 169.5 U/mg	*Aspergillus niger*, mesophilic fungi			[40]
Oil palm empty fruit bunch	0.195 U/g	*Trichoderma* sp.1	Erlenmeyer flasks (250 mL)	3 g	[33]
	0.211 U/g	*Hypocrea neorufa.1*			
Soybean meal	72.6 ± 2.4 U/g	*Yarrowia lipolytica* IMUFRJ50682	Cylindrical polypropylene bioreactors	10 g	[69]
Canola cake	93.9 ± 2.9 U/g				
Wheat bran and sugarcane bagasse	265 U/g	*Rhizopus microsporus* CPQBA 312–07 DRM	Laboratory column bioreactor	10 g	[73]
	113 U/g		Pilot packed-bed bioreactor	15 kg	
Rice bran, rice husk with olive oil	38.67 U/g	*Aspergillus niger*	Erlenmeyer flasks (250 mL)	20 g	[27]
Olive pomace and wheat bran	90.5 ± 1.5 U/g	*Aspergillus ibericus* MUM 03.49, *A. niger* MUM 03.58, *A. tubingensis* MUM 06.152	Erlenmeyer flasks (500 mL)	30 g	[70]
Olive pomace and wheat bran	223 ± 5 U/gds	*Aspergillus ibericus* MUM 03.49	Erlenmeyer flasks (500 mL)	30 g	[19]
			Packed-bed bioreactor	25 g	
Palm kernel cake	–	*Trichoderma viride* SDTC EDF 002	Erlenmeyer flasks (500 mL)	30 g	[71]
	44.43 U/g	*Aspergillus niger* SDTC SRW-4			
	42.05 U/g	*Aspergillus niger* DSMZ 2466			
Rice bran with glycerol	19.844 U/g	*Aspergillus niger*	Cylindrical flasks (500 mL)	40 g	[72]
Rice bran	13.267 U/g				
Rice husk, soybean meal, wheat bran					
Palm kernel cake	34.20 U/gds	*Rhizopus* sp.	Erlenmeyer flasks (250 mL)	50 g	[74]
Olive pomace with wheat bran	120 U/g	*Aspergillus ibericus* MUM 03.49	Tray-type bioreactors	300–500 g	[16]

45–50% non-starch carbohydrates) for rapid growth [56, 64], so that high lipase activities are possible (e.g., 223 U/gds by *Aspergillus ibericus* on olive pomace and wheat bran [19]).

3.1.4 Pectinase

Pectinases are used to break down pectin. Pectin is a common heterogeneous polysaccharide of galacturonic acids with varying proportions of D-galactosyl, L-arabinosyl, or L-rhamnosyl residues in the plant kingdom [53]. Pectinases are subdivided into polygalacturonases, galacturonases, and pectin methylesterases as well as pectin and polygalacturonate lyases.

Just as in the production of amylases, proteases, and lipases, basidiomycetes play only a minor role in the production of pectinases. Pectinases are mainly produced by various plant pathogenic fungi or bacteria [75]. The main application field is the clarification of fruit juices, which has been carried out since about 1930 [53]. Here, further side effects are viscosity reduction and increased fruit juice yields. However, pectinases are also involved in olive oil production, in the production of fruit and vegetable purées, in the treatment of sugar beets, and in the fermentation of apple cider and coffee and cocoa beans. In addition to their use in the food industry, pectinases are mainly used in the textile industry for washing textiles as well as in detergents for dishwashers, in the production of vegetable fibers, and for the treatment of wastewater in the paper and pulp industry [53, 54].

For the production of pectinases on solid substrates, various ascomycetes, such as *Aspergillus* sp. or *Fusarium* sp., have been the focus of recent research (see Table 4). A broad spectrum of usable substrates was found to exist. The highest enzyme activities have been achieved, inter alia, on fruit or vegetable peels. However, studies by Kaur et al. [10] showed that not all fruit and vegetable residues are equally suitable. By comparing different studies, it was found that orange peels in particular appear to be suitable for the production of pectinases (e.g., 3,315 U/gds produced by *Bacillus subtilis* [10]). Wheat bran was also successfully used in pectinase production (e.g., 1828 U/gds produced by *Bacillus tequilensis* [76]). With a mix of different citrus peels and wheat bran, up to 73,000 U/gds of endopectinase activity was achieved [17]. The good suitability of citrus peels can be attributed to their high pectin content of up to 30% [77, 78], which may induce enzyme formation. Wheat bran also has a high fiber content (hemicellulose, cellulose, and pectin) of about 45% [56, 64]. In comparison, apples (whole fruit), for example, contain only 0.5–1.5% pectin [78, 79].

3.2 Lignocellulolytic Enzymes

Lignocellulolytic enzymes include cellulolytic enzymes such as cellulases and xylanases as well as lignolytic enzymes such as laccases and various peroxidases. In nature, these enzymes are formed for nutrient supply through the degradation of lignocellulose, the scaffold of lignified plants, by various microorganisms. Among the best-known representatives are the so-called white rot and brown rot producers, which a large portion of basidiomycetes belong to [75].

Table 4 Review of recent studies on pectinase production on solid substrates (bold: best substrate) including achieved pectinase activity, used production strain, and cultivation size [10, 15, 17, 20, 50, 76, 80–85]

Substrate	Activity	Microorganism	Reactor		Ref.
Jojoba mill solid waste	656.6 U/gds	*Aspergillus oryzae* FK-923			[80]
Tomato processing by-products	53.57 U/mL	*Fusarium solani pisi*	Erlenmeyer flasks (50 mL)	2 g	[81]
Soybean meal, sunflower meal, maize bran, maize pericarp, olive oil cake, wheat bran	64.90 IU/g	*Bacillus* sp. TMF-1	Erlenmeyer flasks (150 mL)	5 g	[50]
Orange peel and coconut fiber agro-residues: wheat bran, rice bran, paddy straw, corn cob, sugarcane bagasse, mustard oil cake, sawdust, mustard straw, cotton straw, groundnut peel, wheat straw, cotton-seed oil cake Fruit peel wastes: lemon peel, mosambi peel, pineapple peel, papaya peel, banana peel, mango peel, coconut fiber, pomegranate peel, orange peel, cheeku peel, kinnow peel	3,315 U/gds (pectinase) 10.5 U/gds (pectin lyase)	*Bacillus subtilis* SAV-21	Erlenmeyer flasks (250 mL)	5 g	[10]
Wheat bran, **orange and lemon peel mix**, citrus peel, lemon peel, apple pomace	73 kU/gds (endo-PG) 197 U/gds (PG) 101 U/gds (PGM)	*A. giganteus* NRRL 10, and 11 others *Aspergillus* sp.	Erlenmeyer flasks (100 mL)	5 g	[17]
			Static tray-type bioreactor	100 g	
Green algae (*Ulva lactuca* and *Codium tomentosum*), brown algae (*Dictyopteris polypodioides*, *Sargassum wightii*, and *Dictyopteris divaricata*)	1,432 ± 1.46 U/mg	*Bacillus licheniformis* KIBGE-IB2 bis B4 *Aspergillus flavus* KIBGE-IB34 *Aspergillus terreus* KIBGE-IB35	Flask	10 g	[82]
Wheat bran, rice bran, cotton seed cake, corn cob, coconut cake, ground nut cake	1828.13 U/gds (pectin lyase) 105.55 U/gds (PG)	*Bacillus tequilensis* SV11-UV37	Erlenmeyer flasks (250 mL)	10 g	[76]
Sugarcane bagasse and citrus pulp	33–41 U/g	*A. oryzae* CPQBA 394–12 DRM 01	Glass columns (4 × 21 cm)	8 g	[83]
			Pilot-scale bioreactor	15 kg	
Orange peels	8.0 U/g	*Penicillium minioluteum* N3C2, *and other* ascomycetes	Columns (25 × 4 cm)	20 g (+ 7 g plastic pieces)	[84]
Wheat bran	298 U/gds	*Aspergillus sojae* ATCC 2023	Tray-type solid-state reactor	50–100 g	[85]

(continued)

Table 4 (continued)

Substrate	Activity	Microorganism	Reactor		Ref.
Wheat bran and sugar-cane bagasse	1,840 U/kgds/h	*Aspergillus niger* CH4	Pilot-scale packed-bed bioreactor	12–30 kg	[15]
Wheat bran and sugar-cane bagasse	22 U/g	*Aspergillus niger* CH4	Pilot-scale packed-bed bioreactor (200 L)	27 kg WB + 3 kg SCB	[20]

The use of these enzymes in industry is diverse and is set to continue expanding. To ensure cost-effective enzyme production, continuous development is necessary. This includes research into new enzyme producers as well as suitable substrates.

In own studies, 41 basidiomycetes were investigated for their suitability as enzyme producers (see Fig. 1). The focus was on their ability to produce laccases, peroxidases, cellulases, and xylanases. The screening was carried out in triplicate on malt agar plates (malt extract agar, Roth, Karlsruhe, Germany) with various additives at room temperature (24–26°C). In order to detect the formation of desired enzymes, for lignolytic enzymes Lev-Blue dye (1 g/L), ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; 0.1 g/L) and guaiacol (100 µL/L) and for cellulolytic enzymes CMC (carboxymethyl cellulose; 5 g/L) and beechwood xylan (5 g/L), as well as pine sawdust and beech sawdust (10 g/L), were added to the agar. Lignolytic activity could be observed directly via the development of a colored halo (ABTS, guaiacol) or by decolorization (Lev-Blue). Cellulolytic activity was determined indirectly by staining with a 1% Congo red solution and the formation of a halo. Thus, it was possible to characterize the different basidiomycetes with regard to their enzymatic performances and their growth rate and to use them for further investigations.

3.2.1 Cellulases

Cellulases degrade cellulose, the main component of the plant cell wall. The term cellulases encompasses several different enzymes, including the three enzymes endoglucanase (endo), exoglucanase (exo), and ß-glucosidase (ß-glu). The different enzymes act synergistically and can be secreted into the system as free enzymes or bound as a multienzyme complex (cellulosome). Endoglucanases preferentially hydrolyze amorphous regions of the cellulose, thereby resulting in oligosaccharides, cellobiose, and glucose. Exoglucanases cleave cellobiose from the nonreducing end, while β-glucosidase predominantly hydrolyzes cellobiose [53, 54].

Cellulases are produced by bacteria, fungi, and plants, and the most important producers belong to the species of *Trichoderma*. The most well-known field of application for cellulases is the fermentation of vegetable waste or other renewable raw materials for the production of bioethanol. They are also used in the production of silage. A further application is their use in detergents, where they degrade superficial lint and thus improve the color intensity and softness of laundry. These

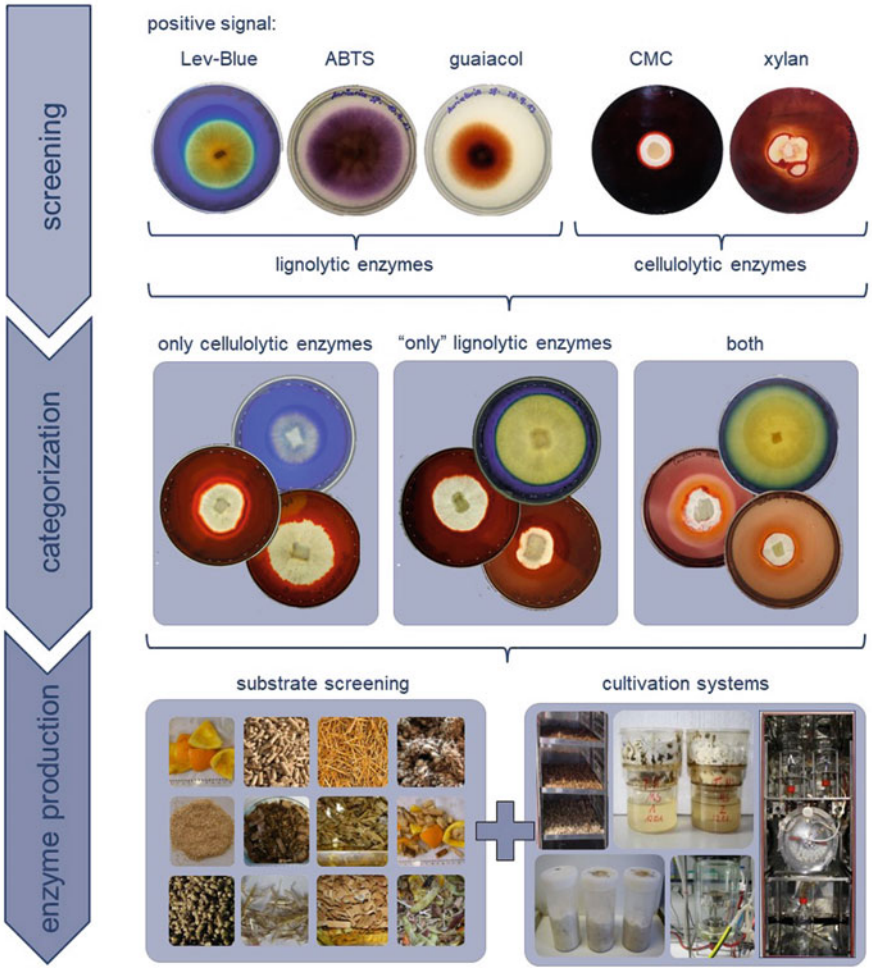


Fig. 1 Depiction of screening and categorization of basidiomycetes with regard to their ability to produce lignocellulolytic enzymes as basis for further investigations

effects are also used in the textile industry, where cellulases are used as softeners, for desizing processes and to produce a stone-wash effect. They are also used in the paper industry for removal of colors from waste paper [53, 54].

In recent studies on the production of cellulases, ascomycetes, such as *Aspergillus* sp. and *Trichoderma* sp., were used [3, 4, 11, 12, 21, 26, 28, 31, 32, 41–43, 48, 84, 86–91]. However, several basidiomycetes, such as *Pleurotus ostreatus* and *Trametes versicolor*, also achieved good results on solid substrates [41, 86, 92]. Residues from the agricultural industry have also been shown to be suitable, such as wheat bran, sugarcane bagasse, or corn straw, which have high levels of cellulose (wheat bran, 15%; sugarcane bagasse, 50%; corn straw, 41%) [93–95] (see Table 5). For example,

Table 5 Review of recent studies on cellulase production on solid substrates (bold: best substrate) including achieved cellulase activity, used production strain, and cultivation size [3–5, 11–13, 21, 26, 28, 31, 32, 41–43, 48, 50, 81, 84, 86–89, 91, 92, 100–103]

Substrate	Activity	Microorganism	Reactor		Ref.
Citrus sinensis bagasse, 12 different agro-industrial wastes	264 U/gds	*Bacillus subtilis*	Flask		[100]
Wheat bran	0.40 IU/g (FPase) 123.64 IU/g (Endo) 18.32 IU/g (ß-Glu)	*Aspergillus oryzae*	Lab-scale bio-reactor, 16 columns (20 × 2.5 cm)		[26]
Carnauba straw, sugarcane, green coconut, cashew	0.9 U/g (FPase) 13 U/g (CMCase)	*Trichoderma reesei* CCT2768	Flasks		[91]
Sugarcane bagasse	11 IU/g (FPase)	*Aspergillus flavus, Trichoderma viride*, and *Pleurotus ostreatus*	Erlenmeyer flasks (250 mL)	2 g	[41]
Tomato processing by-products	39.50 U/mL	*Fusarium solani pisi*	Erlenmeyer flasks (50 mL)	2 g	[81]
Rice by-products: Rice bran, rice husk, and rice straw	1.31 U/gds	*Trichoderma reesei* CECT 2414	Erlenmeyer flasks (150 mL)	5 g	[21]
Wheat bran, wheat straw, rice straw, rice husk, sugarcane tops, sugarcane bagasse, corn cob, carrot grass	26.3 IU/gds (Endo) 2.11 IU/gds (FPase) 34.3 IU/gds (ß-Glu)	*Aspergillus niger* NFCCI	Conical flasks (250 mL)	5 g	[3]
Wheat bran	8 ± 0.2 IU/g (FPase) 17 ± 0.1 IU/g (CMCase) 90 ± 4.0 IU/g (ß-Glu)	*Aspergillus niger* RCKH-3	Erlenmeyer flasks (250 mL)	5 g	[32]
Biomass sorghum and wheat bran	30.64 U/g (Exo) 41.47 U/g (Endo) 54.90 U/g (ß-Glu)	*Aspergillus niger* SCBM1 and *Aspergillus fumigatus* SCBM6	Erlenmeyer flasks (250 mL)	5 g	[43]
Maize bran, almond shells	14.23 IU/gds (5.93 IU/mL)	*Bacillus subtilis* MS 54	Flasks	5 g	[101]
Maize bran, maize pericarp, soybean meal, sunflower meal, olive oil cake, wheat bran	1.19 IU/g	*Bacillus* sp. TMF-1	Erlenmeyer flasks (150 mL)	5 g	[50]
Wheat straw and cotton oil cake Wheat bran, rice bran, mustard oil cake, sugarcane bagasse, sesame oil cake, rice straw, kitchen waste, peanut peel, sawdust	298.12 U/gds (CMCase) 280.13 U/gds (FPase) 6.67 U/gds (ß-Glu)	*Sporotrichum thermophile* BJAMDU5	Erlenmeyer flasks (250 mL)	5 g	[5]
Oil palm frond	2.57 U/g (FPase)	*Aspergillus niger*	Erlenmeyer flasks (250 mL)	5 g	[28]
Corn cob	33.0 U/mL (ß-Glu)	*P. pulmonarius* DBUI002	Erlenmeyer flasks (250 mL)	5 g	[92]
Sawdust	15.0 U/mL (Endo)	*Pleurotus ostreatus* DBUI 14			
Rice bran	13.0 U/mL (Exo)				

(continued)

Table 5 (continued)

Substrate	Activity	Microorganism	Reactor		Ref.
Sweet sorghum bagasse, sweet sorghum stalks, wheat bran, sugarcane bagasse, groundnut shells, corn cobs, rice bran, rice husk, sawdust, groundnut oil cakes, coconut oil cakes, leaf litter	30.32 ± 0.05 U/g	*Trichoderma harzianum* HZN11	Erlenmeyer flasks (250 mL)	10 g	[4]
Forage cactus pear	3456.91 U/g	*Aspergillus niger*	Erlenmeyer flasks (250 mL)	10 g	[89]
	1630.07 U/g	*Rhizopus* sp.			
Rice husks	17.2 ± 0.7 IU/g	*Aspergillus oryzae* ATCC 10124	Erlenmeyer flasks (125 mL)	10 g	[87]
Peanut shells	42.9 ± 0.7 IU/g				
Olive pomace	9.99 ± 1.39 U/g	*Aspergillus* sp. (10 different species)	Erlenmeyer flasks (500 mL)	10 g	[86]
	46 ± 3 U/g	*Trametes versicolor*			
Cassava residue, corncob, rice husk, rice straw, sugarcane bagasse, wheat bran	34.0 ± 2.8 FPU/gds	*Penicillium oxalicum* EU2106	Erlenmeyer flasks (500 mL)	10 g	[102]
Prickly palm cactus husk	4.165 U/mL (CMCase) 30.923 U/mL (FPase)	*Aspergillus niger*	Erlenmeyer flasks	10 g	[88]
	7.859 U/mL (CMCase) 13.571 U/mL (FPase)	*Rhizopus* sp.			
Sawdust	22.97 U/g (ß-Glu)	*Aspergillus* sp.,	Erlenmeyer flasks (250 mL)	15 g	[48]
	138.77 U/g (Endo) 32.16 U/g (Exo)	*Bacillus* sp.			
		Brevibacillus sp.			
Oil palm fronds	12 IU/g	*Aspergillus* sp. and *Trichoderma* sp.	Steriplan™ petri dish (100 × 15 mm)	15 g	[42]
Orange peels	6.5 U/g	*Penicillium minioluteum* N3C2, *Trichoderma reesei* ATCC 26921, *Fusarium* sp. N6C6, *Cladosporium oxysporum* N1C1, *Mucor racemosus* N9C1	Columns (25 × 4 cm)	20 g (+ 7 g plastic pieces)	[84]
Wheat bran	959.53 IU/gds	*Trichoderma reesei* RUT C-30	Erlenmeyer flasks (250 mL)	5 g	[11]
	457 IU/gds		Tray reactor	50 g	
Wheat straw	8.583 ± 0.025 IU	*Trichoderma viride*	Conical flasks (1,000 mL)	100 g	[31]
Agave atrovirens fibers	12860.8 U/g (Endo) 3144.4 U/g (Exo) 384.4 U/g (ß-Glu)	*Trichoderma* spp.	Tray reactor	200 g	[12]

(continued)

Table 5 (continued)

Substrate	Activity	Microorganism	Reactor		Ref.
Wheat bran	375 IU/gds (CMCase) 695 IU/gds (ß-Glu)	*Trichoderma citrinoviride* AUKAR04	Shallow aluminum tray	500 g	[13]
Coffee husk Wood chips were added as bulking agent		*Specialized consortium* (compost)	Erlenmeyer flasks (500 mL)	90 g	[14]
	10 FPU/gds		Packed-bed reactor (4,5 L)	1,2 kg	
	48 ± 4 FPU/gds		Packed-bed reactors (50 L)	15,2 kg	

959.5 IU/gds was obtained by *Trichoderma reesei* RUT C-30 on wheat bran [11]. However, enzyme activities were usually found to be far lower.

Interestingly, very high levels of enzyme activity were found to occur on special substrates such as forage cactus pears and agave atrovirens fibers, with 3,457 U/g (*Aspergillus niger* on forage cactus pears) [89] and 12,861 U/g (endo) and 3,144 U/g (exo) (*Trichoderma* sp. on agave fibers) [12]. Both substrates are succulents and rich in trace elements, vitamins, and dietary fibers. Agave leaf fibers contain 70–80% cellulose and have a low hemicellulose content of 3–30% and low lignification [96]. In comparison, wheat bran has a cellulose content of only 15% and a hemicellulose content of 38% [93]. Competition between the formation of cellulases and hemicellulases is thus probably shifted in favor of cellulases when using succulent fibers.

In our investigations, 14 basidiomycetes were tested on different substrate combinations. Round cups (diameter 4 cm, height 11.5 cm, membrane cap) were filled with 35 g of substrate, made up of one part beech wood chips to one part either hemp litter, corn cob bedding, cotton seeds, or wheat straw. The highest cellulase activity of 627 U/L was achieved by *Flammulina velutipes*, followed by *Lentinus tigrinus* with 603 U/L and *Gloeophyllum trabeum* with almost 500 U/L. The preferred substrate in this case was the mixture of beech wood chips and corn cob bedding when cultivating the two brown rot fungi *F. velutipes* and *G. trabeum*, while the white rot fungus *L. tigrinus* preferred a mixture of beech wood chips and hemp litter for the production of cellulases. Corn cob bedding, with a cellulose content of around 35% [97], and beech wood chips with around 47% [98], have a similar composition to usual suitable substrates, such as wheat bran. In a further experiment with pure corn cob bedding (25 g in 500 mL Erlenmeyer flask) with *F. velutipes* and *Trametes hirsuta*, cellulase activity was even increased to 1,642 U/L for *F. velutipes*. The cellulase activity of *T. hirsuta* was only 382 U/L.

Unlike corn cob bedding, hemp litter has a high cellulose content of up to 70% [99], but the use of hemp litter as an additive only produced good cellulase activities for *L. tigrinus*. This shows how important it is to not only find a suitable producer but also its preferred substrate. For example, *T. hirsuta* was cultivated on 3.5 kg pine wood chips or corn silage in an SSF reactor (rotating drum, working volume 10 L, developed by the Research Center for Medical Technology and Biotechnology,

fzmb GmbH, Bad Langensalza, Germany). Despite the theoretical suitability of corn silage for the production of cellulases, hardly any cellulase activity (0.33 U/L on pine wood chips, 1.2 U/L on corn silage) was observed.

3.2.2 Xylanase

Xylanases, like cellulases, encompass several distinct enzymes. They hydrolyze xylan, the most abundant hemicellulose. Xylanases include endoxylanases (hydrolysis of glycosidic linkages in xylan backbone), β-xylosidases (cleavage of xylose at nonreducing end), α-L-arabinofuranosidases, α-glucuronidases, and acetyl xylan esterase (cleavage of side chains). The main xylanase producers are filamentous fungi, such as *Aspergillus* sp., *Penicillium* sp., *Humicola* sp., and *Trichoderma* sp. In industry, xylanases are produced by submerged fermentation. The induction takes place using insoluble carbon sources, such as xylan, xylobiose, or sophorose [54].

The main application of xylanases is in the animal feed industry. The enzymes ensure a better digestibility of animal feed, especially for pig and poultry farming. They are also used in the food industry to degrade arabinoxylan, thereby increasing yields in grain flour production, improving processability of doughs, and reducing water requirements. In addition, xylanases are used in the extraction of coffee, oils, and starches, as well as in combination with pectinases for the clarification of fruit juices. Other fields of use are the production of fibers from flax, jute, and hemp, as well as the production of cellulose fibers in the paper and pulp industry and the treatment of the resulting wastewater [53, 54].

In recent research, ascomycetes such as *Aspergillus* sp. and *Trichoderma* sp. remain the preferred producers of xylanases. Cultivation is carried out with residues from the agricultural industry, preferably bran or straw (see Table 6). Wheat bran, wheat straw, and corn cobs seem to stimulate xylanase production the most. Xylanase activities of 55,000 IU/gds on wheat bran by *Trichoderma citrinoviride* AUKAR04 [13] or 2,919 U/g by *Aspergillus niger* CCUG33991 [104], as well as 1,189 U/gds on wheat straw by *Botryotinia fuckeliana* CECT 20518 [46] and 795 U/g on corn cobs by *Sporotrichum thermophile* BJAMDU5 [5] and 587 U/gds by *Rhizomucor pusillus* [105], were achieved. Another interesting result was achieved with the use of empty oil palm bunches, on which *Aspergillus niger* USM SD2 yielded xylanase activities of 3,246 IU/gds [106]. No direct conclusions can be drawn regarding substrate composition. The hemicellulose contents vary between 20% (wheat straw) and 43% (corn cob), while the cellulose content is between 24% (wheat bran) and 65% (oil palm empty bunches) with a lignin content (with the exception of corn cobs 5%) between 13% and 27% [64, 94, 97, 107].

As already described for the investigations on cellulase formation, 14 basidiomycetes were tested on different substrate combinations in round cups (diameter 4 cm, height 11.5 cm, membrane cap) filled with 35 g of substrate, consisting of one part beech wood chips to one part either hemp litter, corn cob bedding, cotton seeds, or

Table 6 Review of recent studies on xylanase production on solid substrates (bold: best substrate) including achieved xylanase activity, used production strain, and cultivation size [5, 6, 13, 23, 28, 41–43, 46, 48, 84, 86, 89, 91, 104, 105, 108–110]

Substrate	Activity	Microorganism	Reactor		Ref.
Corn bran mixed with polyurethane as support (75:25), wheat bran, coffee pulp	33.4 U/gds (acetyl xylan esterase)	*Aspergillus niger* PCS6	Glass columns		[108]
Carnauba straw, sugarcane, green coconut, cashew	99.5 U/g	*Trichoderma reesei* CCT2768	Flasks		[91]
Cocoa meal	72 U/g	*Aspergillus awamori* IOC-3914			[109]
Oil palm empty bunches	3,246 IU/gds	*Aspergillus niger* USM SD2			[106]
Sugarcane bagasse	180 IU/g	*Aspergillus flavus*, *Trichoderma viride*, and *Pleurotus ostreatus*	Erlenmeyer flasks (250 mL)	2 g	[41]
Corn cob, agro-industrial wastes	587.6 U/gds (packed-bed)	*Rhizomucor pusillus*	Tray bioreactor and packed-bed bioreactor	3 g	[105]
Biomass sorghum and wheat bran	300.07 U/g (Xyl) 41.47 U/g (ß-Xyl)	*Aspergillus niger* SCBM1 and *Aspergillus fumigatus* SCBM6	Erlenmeyer flasks (250 mL)	5 g	[43]
Wheat straw and cotton oil cake Wheat bran, rice bran, mustard oil cake, sugarcane bagasse, sesame oil cake, rice straw, kitchen waste, peanut peel, sawdust	795.12 U/g	*Sporotrichum thermophile* BJAMDU5	Erlenmeyer flasks (250 mL)	5 g	[5]
Wheat straw, grape pomace, orange peels, rice husk	1,189 U/gds	*Botryotinia fuckeliana* CECT 20518, *Aspergillus awamori* CECT 2907, *Trichoderma reesei* CECT 2414, *Phanerochaete chrysosporium* CECT 2798	Petri dishes	5 g	[46]
Oil palm frond	4.12 U/g	*Aspergillus niger*	Erlenmeyer flasks (250 mL)	5 g	[28]
Olive pomace	19.44 ± 3.85 U/g	*Aspergillus* sp. (ten different species)	Erlenmeyer flasks (500 mL)	10 g	[86]
	100 ± 15 U/g	*Trametes versicolor*			
Wheat bran, rice bran, sugarcane molasses, cellulose paper powder	24.66 U/mL	*Streptomyces hygroscopicus*	Erlenmeyer flasks (250 mL)	10 g	[110]
Wheat bran, sugarcane bagasse, rice bran, corn cob, wheat straw, sawdust	139 U/mL	*Aspergillus* spp.	Erlenmeyer flasks (250 mL)	10 g	[6]
Forage cactus pear	355.56 U/g	*Aspergillus niger*	Erlenmeyer flasks (250 mL)	10 g	[89]
	204.57 U/g	*Rhizopus* sp.			

(continued)

Table 6 (continued)

Substrate	Activity	Microorganism	Reactor		Ref.
Oil palm fronds	109 IU/g	*Aspergillus* sp. and *Trichoderma* sp.	Steriplan™ petri dish (100 × 15 mm)	15 g	[42]
Sawdust	104.96 U/g	*Bacillus* sp., *Aspergillus* sp., *Brevibacillus* sp.	Erlenmeyer flasks (250 mL)	15 g	[48]
Corn cob, agro-wastes	12.30–48.63 U/g	*Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Trichoderma longibrachiatum*, *Botryodiplodia* sp.	Erlenmeyer flasks (250 mL)	25 g	[23]
Orange peels	16.5 U/g	*Penicillium minioluteum* N3C2, *Trichoderma reesei* ATCC 26921, *Fusarium* sp. N6C6, *Cladosporium oxysporum* N1C1, *Mucor racemosus* N9C1	Columns (25 × 4 cm)	20 g (+ 7 g plastic pieces)	[84]
Wheat bran, sorghum stover, corn cob, and soybean meal	2,919 ± 174 U/g	*Aspergillus niger* CCUG33991	Erlenmeyer flasks (250 mL)	5 g	[104]
			Tray bioreactor	100 g	
Wheat bran	55,000 IU/gds	*Trichoderma citrinoviride* AUKAR04	Shallow aluminum tray	500 g	[13]

wheat straw. By far the highest xylanase activities, 23,063 U/L and 7,344 U/L, were achieved by *F. velutipes* and by *Schizophyllum commune*, both brown rot fungi. Here, the preferred substrate was also a mixture of beech wood chips and corn cob bedding, a substrate already classified as suitable in the literature [23, 104, 105]. The good suitability of *F. velutipes* for the production of xylanases, which was at the same level of known producer strains [13, 104, 106], was also confirmed in subsequent experiments with pure corn cob bedding (25 g in 500 mL Erlenmeyer flask). In this case, xylanase activity of 16,739 U/L was achieved.

3.2.3 Laccases and Peroxidases

Lignin is a heterogeneous biopolymer consisting of aromatic phenolic building blocks in the plant cell wall. It causes lignification and thus an increase in the stability of plant material. Lignin degradation is very slow due to its high complexity, and it is preferably carried out by representatives of white rot fungi, especially basidiomycetes. It is an oxidative process and cannot be carried out by a single enzyme, so a consortium of different enzymes that interact with each other is needed. These include oxidative enzymes such as laccases, peroxidases, lignin peroxidases, and manganese peroxidases [75].

Laccases belong to the multicopper oxidases and are mainly formed by filamentous fungi. These include, in particular, various basidiomycetes, such as *Agaricus bisporus*, *Cerrena unicolor*, and *Trametes versicolor*. They are used in the pulp and paper industry (e.g., paper bleaching), the textile industry (e.g., denim bleaching), cosmetics (e.g., hair bleaching), the wood industry, the food industry, the production of biofuels, and the pharmaceutical industry (e.g., transformation of antibiotics) [111].

Peroxidases are a diverse group of enzymes that reduce peroxide, usually hydrogen peroxide. Manganese and lignin peroxidases as well as non-specific peroxidases, mainly produced by fungi, are used especially for lignin degradation [75].

As already mentioned above, the main producers of lignolytic enzymes are filamentous fungi, especially the basidiomycetes. This is also reflected in the organisms studied for the production of these enzymes on various solid substrates in the literature. Again, various residues from the agricultural industry were used (see Table 7).

Substrates with a high lignin content were popular, in other words lignified materials, such as sawdust or vineyard trimmings. However, only moderate enzyme activities were achieved, for example, 305 U/gds on sawdust by *Fusarium equiseti* VKF-2 [35]. The use of fruit peels or leaves proved much more successful. For example, 1,200 U/g was observed for laccase activity of *Phanerochaete chrysosporium* CECT 2798 on orange peel [46] and 1,633 U/ml of *Pleurotus ostreatus* strain NCIM 1200 on pineapple leaves [112], as well as 10,800 U/L for peroxidase activity of *Pleurotus eryngii* IJFM 169 on banana peels [9]. Also, the use of pretreated substrates, such as steam-exploded corn stalks (2,600 U/g laccase activity of *T. versicolor* [45]), or *Parthenium* sp., a belligerent noxious herbaceous wasteland weed (34,444 U/gds laccase activity of *Pseudolagarobasidium acaciicola* [30]), showed a considerable increase in laccase activity. This effect may be attributed to the availability of vegetable ingredients, such as essential oils in orange peels or parthenolide in the wasteland weed, whose aromatic structures similar to the degradation products of lignin may promote the formation of laccases and peroxidases.

In addition to known basidiomycetes, such as *Pleurotus eryngii* [9] and *Marasmius* sp. [114], two fungi species, *Lentinus tigrinus* and *Trametes hirsuta*, were also investigated in our studies due to the good suitability of basidiomycetes, especially white rot fungi, for the production of lignolytic enzymes.

First investigations in round cups (diameter 4 cm, height 11.5 cm, membrane cap) filled with 35 g of substrate, consisting of cotton seeds, pine wood chips or wheat straw, showed the investigated fungi (*P. eryngii*, *Marasmius* sp., *L. tigrinus*) to be suitable for laccase formation in principle. As in the studies on cellulase and xylanase activity, the activities achieved differed depending on the producer and substrate used. However, it was found that by far the highest activity of laccases for all three fungi tested was achieved on wheat straw (*P. eryngii*, 131 U/L; *Marasmius* sp., 117 U/L; *L. tigrinus*, 70 U/L). The lignin content of wheat straw with max. 25%

Table 7 Review of recent studies on production of lignolytic enzymes on solid substrates (bold: best substrate) including achieved activity, used production strain, and cultivation size [8, 9, 30, 35, 41, 42, 44–46, 48, 112–114]

Substrate	Activity	Microorganism	Reactor		Ref.
Laccase					
Sawdust, groundnut oil cake, neem oil cake, rice bran	305 U/gds	*Fusarium equiseti* VKF-2	Flask		[35]
Peanut shell	~5 U/g	*Pycnoporus* sp. SYBC-L3	Flasks (250 mL)		[113]
Vineyard trimmings	0.34 mU/mg protein	*Lentinus edodes*			[44]
Wheat straw	0.08 mU/mg protein				
Sugarcane bagasse	10 IU/g	*Aspergillus flavus*, *Trichoderma viride*, and *Pleurotus ostreatus*	Erlenmeyer flasks (250 mL)	2 g	[41]
Peels of citrus fruits, soybean meal, tofu dreg, lignin monomers, tea leaves, peels of onion and kiwi, paper, dying industry effluents		*Lysinibacillus* sp.	Flasks (250 mL)	5 g	[8]
Steam-exploded corn stalk	2600.33 ± 81.89 U/g	*Trametes versicolor*	Erlenmeyer flasks (250 mL)	5 g	[45]
Rice bran, rice straw, sugarcane bagasse, sawdust, pigeon pea waste	1.4 U/mL	*Marasmius* sp. BBKAV79	Conical flasks (250 mL)	5 g	[114]
***Parthenium* sp.** (belligerent noxious herbaceous wasteland weed), rice straw, wheat straw, sugarcane bagasse	34,444 U/gds	*Pseudolagarobasidium acaciicola* LA 1	Erlenmeyer flasks (250 mL)	5 g	[30]
Orange peels, wheat straw, grape pomace, rice husk	1,200 U/g	***Phanerochaete chrysosporium*** CECT 2798, *Botryotinia fuckeliana* CECT 20518, *Aspergillus awamori* CECT 2907, *Trichoderma reesei* CECT 2414	Petri dishes	5 g	[46]

(continued)

Table 7 (continued)

Substrate	Activity	Microorganism	Reactor		Ref.
Pineapple leaves	1632.63 IU/mL	*Pleurotus ostreatus* strain NCIM 1200	Erlenmeyer flasks (250 mL)	5–25 g	[112]
Sawdust	71.18 U/g	***Bacillus* sp.**, *Aspergillus* sp., *Brevibacillus* sp.	Erlenmeyer flasks (250 mL)	15 g	[48]
Peroxidase					
Banana peel	10,800 U/L 36 U/gds	*Pleurotus eryngii* IJFM 169	Erlenmeyer flasks (250 mL)	6 g	[9]
Sawdust	729.12 U/g	***Aspergillus* sp.**, *Bacillus* sp., *Brevibacillus* sp.	Erlenmeyer flasks (250 mL)	15 g	[48]
Manganese peroxidase					
Vineyard trimmings	2.21 mU/mg protein	*Lentinus edodes*			[44]
Wheat straw	0.60 mU/mg protein				
Sawdust	47.73 U/g	***Bacillus* sp.**, *Aspergillus* sp., *Brevibacillus* sp.	Erlenmeyer flasks (250 mL)	15 g	[48]
Lignin peroxidase					
Oil palm fronds	222 IU/G	*Aspergillus* sp. and *Trichoderma sp.*	Steriplan™ petri dish (100 × 15 mm)	15 g	[42]

and 9% of cotton seeds is lower than that of pinewood chips, which is around 27% [94, 115, 116]. The lower lignin content and the simplified accessibility might favor laccase production on wheat straw or cotton seeds, as opposed to the use of untreated pinewood chips.

In a further study, the versatile white rot fungi *T. hirsuta* was cultivated on various substrates in an SSF reactor (rotating drum, working volume 10 L, developed by the Research Center for Medical Technology and Biotechnology, fzmb GmbH, Bad Langensalza, Germany) (see Table 8).

Once again, enzyme activity was confirmed to be strongly dependent on the substrate used. It was shown again that substrates with high lignin content, such as pinewood chips, do not necessarily result in better enzyme production. In contrast, substrate mixtures can significantly improve enzyme production. Thus, the positive effect on enzyme formation when using orange peels, as also described in the literature [46], was confirmed and amplified by the mixture with pinewood chips, leading to greater structural integrity and improved air exchange in the substrate bed (compare laccase activity in Table 8).

Table 8 Comparison of lignolytic enzyme activities achieved by *T. hirsuta* cultivated on different substrates in an SSF rotating drum reactor

Substrate		Pinewood chips	Orange peels	Pinewood chips + orange peel + xylidine	Corn silage
Enzyme					
Laccase	U/L	106	742.5	1,881	346
Unsp. peroxidase	U/L	11.4	99.8	433	51
Manganese peroxidase	U/L	5.6	–	24.37	473
Lignin peroxidase	mU/L	4	–	6.5	153

4 Conclusions

Solid-state fermentation is a cost-effective and sustainable alternative for the production of technically relevant extracellular enzymes by selected filamentous fungi and bacteria on industrial residues. This review has shown that, in addition to the engineering aspects, the combination of producer strain and substrate is crucial for effective enzyme production.

The substrates used were mainly residues from the agricultural industry, the food industry, and the forestry. In most cases, the choice of substrate was made based on its composition and the natural function of the target enzyme. For example, starch-containing substrates were preferably used for the production of amylases. However, the example of lignolytic enzymes showed that it is not always sufficient to make the component that is to be degraded available in large quantities. It was found that ease of accessibility is also a significant factor in ensuring good induction of enzyme formation.

Wheat bran, either alone (e.g., for the production of amylases) or in combination with other substrates (e.g., for the production of lipases), is a very versatile and popular substrate for enzyme production. Various types of bran, as well as straw and fruit peel, were found to be suitable for the production of amylases despite their low starch content. As expected, proteases were preferably produced on protein-rich substrates, such as soy residues, wheat and rice bran or animal-origin residues such as cuttlefish. Lipid-rich substrates, such as coconut meal, olive pomace, and palm kernel cake, partly in combination with wheat bran, were successfully used for lipase production. For the formation of pectinase, citrus fruit peels were especially suitable. Wheat bran, sugarcane bagasse, and corn straw are often used for cellulase production. However, it turned out that especially the use of succulent fibers, such as forage cactus pears and agave atrovirens fibers, had a very positive effect on the formation of cellulases. For the formation of xylanases, a very diverse spectrum of substrates could be used, such as wheat bran, wheat straw and corn cobs, empty oil palm bunches, and corn cob bedding. Lignolytic enzymes, such as laccases and various peroxidases, were formed primarily on substrates with relatively readily available

aromatic compounds, such as fruit peels (e.g., orange peels) or leaves, and not, as expected, on substrates with very high lignin contents.

Finally, it was shown that there is not just one ideal substrate for each enzyme but that the choice of substrate needs to take into account the preferred substrate of the producer chosen for the enzyme.

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Part II
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Aroma Profile Analyses of Filamentous Fungi Cultivated on Solid Substrates

Axel Orban, Marco A. Fraatz, and Martin Rühl

Contents

Abstract Filamentous fungi have been used since centuries in the production of food by means of solid substrate fermentation (SSF). The most applied SSF involving fungi is the cultivation of mushrooms, e.g., on tree stumps or sawdust, for human consumption. However, filamentous fungi are also key players during manufacturing of several processed foods, like mold cheese, tempeh, soy sauce, and sake. In addition to their nutritive values, these foods are widely consumed due to their pleasant flavors. Based on the potentials of filamentous fungi to grow on solid substrates and to produce valuable aroma compounds, in recent decades, several studies concentrated on the production of aroma compounds with SSF, turning

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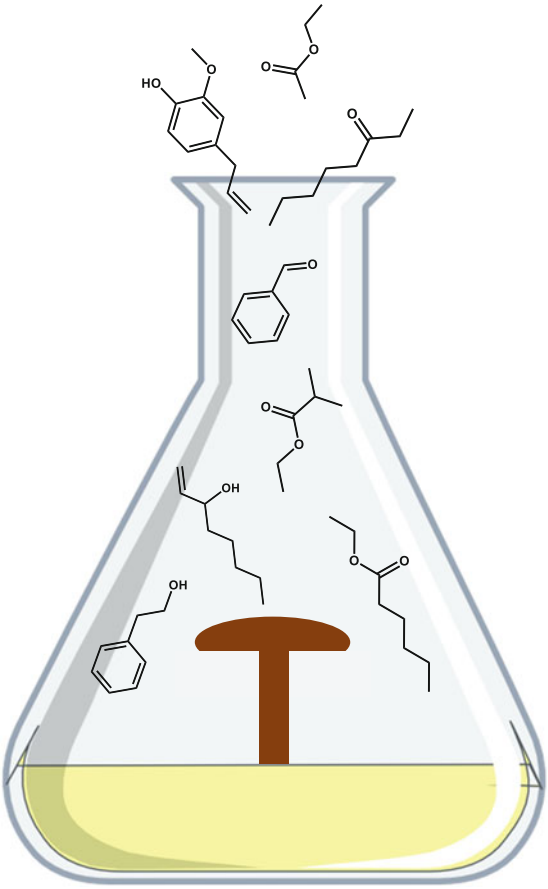
cheap agricultural wastes into valuable flavors. In this review, we focus on the presentation of common analytical methods for volatile substances and highlight various applications of SSF of filamentous fungi dealing with the production of aroma compounds.

Graphical Abstract



Keywords Ascomycetes, Basidiomycetes, Fermented food, SSF, Volatile organic compounds

Abbreviations

6-PP	6-Pentyl-α-pyrone
AAO	Aryl alcohol oxidase(s)
ADA	Aroma dilution analysis
AEDA	Aroma extract dilution analysis
CAR	Carboxen
DHS	Dynamic headspace
DM	Dry matter
DVB	Divinylbenzene
FD	Flavor dilution factor
FID	Flame ionization detector
GC	Gas chromatography
HS	Headspace
LLE	Liquid-liquid extraction
MS	Mass spectrometer
O	Olfactometry
OAV	Odor activity value
ODP	Olfactory detection port
PA	Polyacrylate
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
SAFE	Solvent-assisted flavor evaporation
SBSE	Stir bar sorptive extraction
SmF	Submerged fermentation
SPME	Solid-phase microextraction
SSF	Solid substrate fermentation
TD	Thermal desorption
VOC	Volatile organic compound(s)

1 Aroma Profile Analysis

The analysis of aroma active compounds in food started in the 1960s with the possibility to separate complex aroma mixtures by means of capillary gas chromatography (GC) (reviewed in [1]). With this analytical method, new possibilities arose to determine the volatile composition of a sample. Nevertheless, the impact of each volatile organic compound (VOC) on the human nose still remained unknown until GC-olfactometry (GC-O) entered the aroma research. GC-O enables the correlation between a VOC and its perception using the human nose as a detector [1–3]. In most cases, the outlet of the GC capillary column is installed into a column flow splitter. The column flow splitter possesses two outlets directing the gas flow into a destructive detector (e.g., flame ionization detector

Fig. 1 GC-MS-O equipped with an autosampler and an ODP (highlighted)

(FID) or mass spectrometer (MS)) and into a nondestructive olfactory detection port (ODP) (Fig. 1). With this equipment, it is possible to directly assign an odor impression to an MS spectrum or FID peak, respectively [3, 4].

1.1 Methods for Volatile Organic Compound Extraction

Prior to the GC analysis, VOC have to be extracted from the analyte's matrix. Among others, this can be food, a plant, or a microbial culture, and the method of extraction depends on the matrix as well as on the VOC to be analyzed.

1.1.1 Solvent Extraction

Several extraction methods for aroma analysis exist, each having different pros and cons. This makes the decision for an optimal analytical method difficult. Continuous extraction of volatile organic compounds from a solid matrix using a Soxhlet extractor followed by a concentration of the extract is a basic method for

gaining an aroma extract. In 1964, Likens and Nickerson improved this method by inventing an apparatus for simultaneous solvent extraction and distillation, reducing the thermal load on the sample [5]. Nevertheless, elevated temperatures during distillation and extraction may lead to artefact formation. Thermal stress during extraction can be overcome by using a liquid-liquid extraction (LLE). Generally, the solid matrix is dispersed in an aqueous phase and extracted exhaustively using an organic solvent. For an efficient extraction and the subsequent analysis, different properties of the solvent including polarity, density, solubility, and potential reactivity regarding the analytes have to be taken into account [5]. The disadvantage of LLE is the often occurring concurrent extraction of nonvolatile compounds resulting in difficulties during GC analysis. This disadvantage as well as thermal stress can be reduced to a minimum by applying the solvent-assisted flavor evaporation (SAFE) method. The core part of this procedure is the SAFE distillation unit [6], which is evacuated by a high vacuum pump. The distillation starts by dropping the sample extract into the evaporation flask. The immediately formed vapor is transported into the distillation head, where nonvolatile compounds are trapped. Volatile compounds condense in a sample collection flask, which is cooled in a Dewar using liquid nitrogen [6]. Nonetheless, the demand for environment-friendly as well as more easy and fast extraction methods led to the development of solvent-free procedures.

1.1.2 Dynamic Headspace

One of the first dynamic headspace (DHS) systems was developed in the 1970s to establish a routine procedure for volatile extraction and analysis by means of GC [7]. DHS involves passing a defined flow of (inert) gas through a container holding the sample. The VOC inside the container are carried by the steady gas flow into a sorbent, a cryogenic container, or a solvent, where they are trapped [8]. When the gas flow is led through a liquid sample, the method is also referred to as “purge and trap” [9]. DHS can be conducted in a circular system by using a closed-looped stripping apparatus, where the extraction gas flows in a closed circuit [10, 11]. The sample might be heated, stirred, or supplemented with salts to increase the volatility of the analytes [9, 12]. With DHS, exhaustive extraction of the sample is possible, since the analytes are permanently removed from the headspace (HS), and therefore no equilibrium between the matrix and the gas phase is established [13]. In addition to the necessity to elicit the right gas flowrate, the extraction time and temperature as well as the type of sorbent in the trap are crucial parameters. Various trapping materials are commercially available, including activated charcoal, poly(2,6-diphenyl-*p*-phenylene oxide) (Tenax®), silica-based materials (Chromosorb®), carbon molecular sieves (Carboxen®), and graphitized carbon (Carbotrap®). Traps containing different types of sorbents are used frequently in order to achieve the extraction of a wider range of substances [14]. Depending on the sorbent applied, desorption of the VOC can be performed with a solvent or thermally in the GC [15]. For thermal desorption (TD) applications, Tenax® is often used due

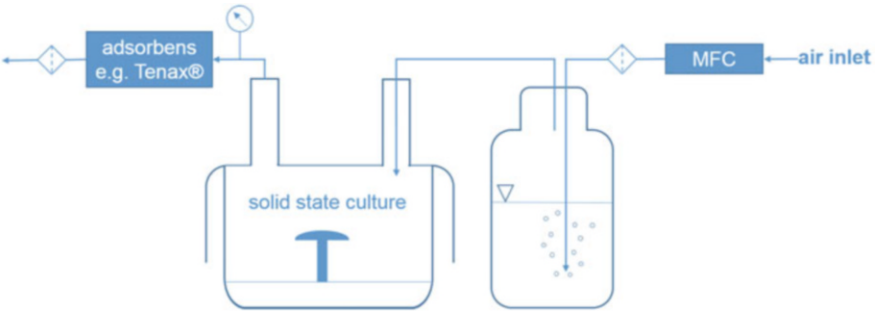


Fig. 2 Scheme of a laboratory installation for DHS during SSF of a fungus. *MFC* mass flow controller

to its high thermostability, its low water adsorption capacity, and its low bleed characteristic [8, 15, 16]. Nonetheless, Tenax® has some disadvantages: e.g., it has only a small surface area, resulting in a low adsorption capacity, and it is limited to the extraction of more nonpolar analytes because of its low affinity for polar compounds [17].

Generally, DHS is an important extraction method for volatile compounds since it reduces or even eliminates the need of solvents. It offers the possibility of multiple trapping approaches as well as the usage of various sorbent materials [18]. During solid substrate fermentation (SSF), DHS is applied in our laboratory to extract VOC produced during the cultivation of filamentous fungi (Fig. 2).

1.1.3 Microextraction Methods SPME and SBSE

Further options for the extraction of volatile compounds comprise solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE) [19]. SPME was presented to the scientific world in the early 1990s as a new possibility to perform sample extractions in a more environment-friendly way [20]. SPME is a non-exhaustive extraction technique in which a fiber coated with sorbent materials is exposed to the sample [20, 21]. Commercial SPME fibers often consist of fused-silica as a carrier modified with different absorbent or adsorbent materials, including polydimethylsiloxane (PDMS), polyethylene glycol (PEG), polyacrylate (PA), divinylbenzene (DVB), carboxen (CAR), as well as combinations thereof [22–24]. This diversity of available coatings makes SPME the method of choice in a broad range of applications, such as in the food [25], aroma [26], medical [27], environmental [28], or bioanalytical [29] sector. Extraction can be performed in the HS by incubating the fiber in the vapor phase above the sample (Fig. 3) or by direct immersion of the fiber into the matrix [30].

The benefits of SPME compared to LLE are the absence of solvents, the higher sensitivity, the demand for less sample material, and a faster and convenient handling [19, 20]. Furthermore, the entire extraction process at a specific

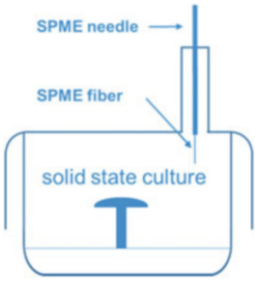


Fig. 3 SPME applied during SSF of a fungus

temperature for a given period can be conducted automatically by using GC systems equipped with multipurpose autosamplers (Fig. 4). Nonetheless, SPME suffers from drawbacks regarding the stability of the fiber and coating, the capacity for analytes, and the durability in organic solvents [23, 31]. It is worth mentioning that new materials for SPME fibers and coatings are on the rise, reducing the aforesaid problems [22, 32].

SBSE was developed around 20 years ago as a new microextraction method [33]. For SBSE, a magnetic stir bar, covered with a layer of sorbent material, usually PDMS [34], is used. In contrast to SPME, the coating is 50–250 times thicker, resulting in a higher capacity and sensitivity [35]. Extraction can be performed in the HS above a sample or, more common, in an aqueous sample by stirring the sample under controlled conditions [36]. The extraction process is followed by the desorption of the analytes which can be accomplished thermally or by rinsing the stir bar with a solvent [37]. In most SBSE GC applications, the TD is preferred due to easier handling. TD is usually conducted at a temperature range of 150–300°C. Compared to SPME, desorption time can be quite long, up to 15 min, due to the thicker coating of the stir bar. In this case, compounds might desorb too slowly, leading to insufficient chromatographic separation of the analytes. To ensure an adequate analysis, cryofocusing of the substances in the GC inlet is necessary [37]. Cryotrapping enables quantitative transfer of the analytes (with considerable increase in sensitivity) and minimizes chromatographic peak width [38]. Besides the need for special equipment, during the early days of SBSE, one drawback was that only PDMS as a coating material was commercially available. This made the analysis less suitable for more polar compounds. In recent years, new PEG- and PA-containing sorbents for SBSE were developed, thus making this method attractive for a broader range of applications [39]. Besides SPME and SBSE, further microextraction techniques like single-drop microextraction and microextraction by packed sorbent offer solutions for a widespread field of analytical problems [28, 40]. It is important to keep in mind that every method leads to a unique aroma profile and that there is no universal extraction technique available [41].

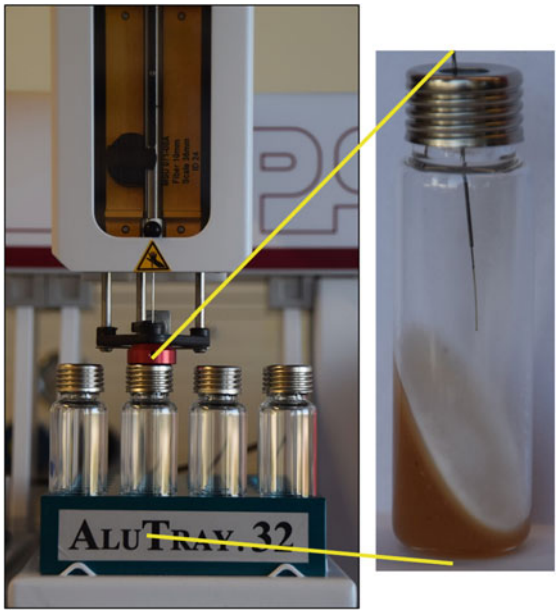


Fig. 4 Automatically performed SPME GC-MS analysis of SSF with filamentous fungi in 20 mL vials (height 7.5 cm; width 2 cm)

1.2 Detection of Volatile Compounds

Generally, the identification of volatile compounds is based on retention indices (RIs) and mass spectral analysis. RIs of the analytes obtained on two columns of different polarity have to be compared with those of the corresponding authentic reference compounds. In addition, the mass spectra of the analytes have to correspond to the mass spectra of the standard substances [42, 43]. Linear RIs modified after Kováts [44] should be calculated according to the retention times of homologous *n*-alkanes by linear interpolation. Additionally, it is common to compare the odor quality of the analyte with that of the authentic standard by olfactory detection at comparable concentration levels [43]. Although single compounds can be identified with this approach, the impact of each VOC on the overall aroma profile is still unknown. Especially in fermented products, several hundreds of VOC are present, i.e., more than 800 in coffee [1, 45]. To determine the compounds representing the sample, the so-called character impact compounds, an aroma extract dilution analysis (AEDA) using GC-O is carried out [45]. A stepwise dilution (generally 2^n) of an extract is performed, and each dilution is analyzed by means of GC-O until no compound is perceived at the ODP [45]. The highest dilution at which a flavor compound can still be perceived is defined as the so-called flavor dilution factor (FD) [45]. Thus, it is assumed that aroma compounds with the highest FD factors mainly contribute to the overall aroma. By focusing on these substances, the key odor compounds can be identified [4, 46]. In recent studies as an alternative to AEDA, an aroma dilution analysis (ADA) is performed without the need of

preparing an extract due to the usage of SPME or SBSE. The stepwise dilution is achieved by adjusting the split ratio of the carrier gas flow [47, 48].

Generally, the FD factor of a VOC is meaningless concerning the impact of this compound on the overall olfaction. For this purpose, the odor activity value (OAV) can be determined [43]. Besides the concentration of a specific compound, the odor threshold of a standard substance of this volatile compound has to be determined by several panelists. The OAV is then calculated by dividing the concentration of the volatile compound in the sample with the determined threshold of the specific substance. Only VOC with an OAV >1 are taken into account.

2 Fungal Volatile Organic Compounds

Higher fungi are a remarkable source of aroma compounds highly valued by humans since ancient times: (1) mushrooms are an esteemed repository for nutrition, appreciated for their medical benefits and their unique as well as delicious flavors, and (2) ascomycetes, like *Saccharomyces cerevisiae*, are essential in food processing, ensuring the quality and taste, e.g., in bread or beer. This review focuses on the aroma compounds produced by filamentous fungi during SSF within various applications. Besides the discussion on different VOC produced, the various extraction methods used within the works listed below should depict the possibilities of aroma analysis during SSF.

2.1 Fungal VOC in Food Manufacturing

Ascomycetes such as *Aspergillus oryzae* and *Aspergillus sojae* have been applied to ferment soy bean, rice, and wheat to hydrolyze starch and proteins. The product of this SSF is called *koji* and is used as a starter culture for subsequent fermentation of the material into soy sauce, miso, or sake [49]. Ito et al. investigated the volatile compounds during the production of *koji* for the sake manufacturing process, which is rice fermented with *A. oryzae* [50]. Husked rice grains were soaked in water for 2 h, steamed for 30 min, cooled, and drained. The rice was inoculated with conidia (asexual spores), and incubation was performed at 36°C with a relative humidity of 95%. Volatiles were extracted using a DHS system equipped with a Tenax® trap, desorbed via TD, and analyzed by means of GC-FID and GC-MS. In total, 17 compounds were identified, including alcohols (e.g., ethanol, 3-methyl-1-butanol, butanol, oct-1-en-3-ol, and octan-1-ol), aldehydes (e.g., acetaldehyde), ketones (e.g., acetone, butanone, and octan-3-one), and the ester ethyl acetate. Their presence within the headspace of the active *koji* culture varied during cultivation. After 22 h in the mid-log phase of the SSF, a grassy impression was dominant, whereas after 38 h, the grassy fragrances decreased, and oct-1-en-3-ol increased resulting in the mushroom-like odor in the stationary phase of *koji* making. The dependence of

Fig. 5 Surface of a 3-day-old *Rhizopus oligosporus* culture on soy beans (left) and cross section of the tempeh (right); photos kindly provided by Andrea Sabbatini

VOC on the stage of *koji* production enables process control of SSF on the basis of VOC analysis. This is depicted in the work of Kum et al. [51], where a soybean-based *koji* paste named *doenjang* is produced by means of SSF over a period of 8 weeks. The authors conducted a principal component analysis of the analyzed VOC, which revealed a distinct clustering of the calculated VOC components in relation to the cultivation period. Another soybean-based food is the traditional East Asian *tempeh*. Generally, for *tempeh* production, soybeans are soaked in water, cooked, and used as solid substrate for fungal colonization by the filamentous zygomycete *Rhizopus oligosporus*. After fermentation a structured matrix is formed, known as *tempeh* (Fig. 5). The aroma of fresh *tempeh* is specified by mainly moldy, mushroom-like, earthy, and boiled potato-like aroma compounds, such as 2-methylpropanal, oct-1-en-3-one, and 3-methylsulfanylpropanal (methional) [52, 53]. LLE of VOC from *tempeh* with subsequent SAFE and GC-MS analysis, followed by the determination of the corresponding OAV, revealed 2-methylpropanal and oct-1-en-3-one as main aroma compounds after 1 day of fermentation. After 5 days of fermentation, the boiled potato odor methional ensued [53]. In a HS analysis of *tempeh* with Tenax® as adsorbent [54], it was not possible to detect methional nor the typical mushroom odor oct-1-en-3-one found in liquid extracts, but other C8 volatiles were present. Interestingly, the key aroma compound 2-methlypropanal was detected in the fermented soybeans as well as in the non-fermented control, although in the latter only in small concentrations [54].

An SSF for food manufacturing with filamentous fungi adapted in Europe is the production of soft cheese including camembert and blue cheese, e.g., Roquefort and Gorgonzola. Filamentous fungi play an important role in the maturation of these cheeses, contributing to the unique flavor, texture, and product composition [55]. Camembert, a mold-ripened cheese originating from France, is traditionally manufactured from raw cow milk [56]. During production, the curd is molded with *Penicillium camemberti* on the surface, imparting the cheese its pronounced flavor. Analysis of the camembert aroma was performed using solvent and static HS extraction by means of GC-MS followed by AEDA and calculation of FD values [57] or OAV [58]. In both works, 42 VOC were identified of which dimethyl sulfide, methional, and especially methanethiol were key odorants of the sulfurous, garlic note. The C8 volatiles oct-1-en-3-one and oct-1-en-3-ol contributed to the

mushroom-like odor, whereas 3-methylbutanal could be linked to the malty scent of the camembert, and 2-phenylethyl acetate was related to a floral bouquet of the cheese. In contrast to the cultivation on curd, no sulfur-containing volatiles were detected when *P. camemberti* was cultivated on potato dextrose agar or Czapek's agar [59]. Similar to the curd fermentation, C8 compounds, including oct-1-en-3-ol, octan-3-ol, and octan-3-one, have been extracted with a DHS device equipped with Tenax® traps and identified in agar plate cultures. Another *Penicillium* species used for blue cheese manufacturing is *P. roqueforti*. Similar to Camembert fabrication, fungal conidia are either directly added to the milk or sprayed on the curd during blue cheese production. Usually, ripening is performed for about 90 days at 10°C and a relative humidity of at least 90% [60, 61]. *P. roqueforti* has a high tolerance regarding physical parameters. It can even grow in an oxygen-poor environment and in a broad pH range of 3–10. This enables, by adjusting suitable conditions, a selective growth of the fungus inside the cheese [62]. During maturation, several peptidases and lipases are expressed by *P. roqueforti*, leading to extensive degradation of proteins (up to 35%) and fats [60, 63, 64]. Portions of the released fatty acids are converted to 2-methyl ketones via oxidation to β-keto acids and a decarboxylation step [65]. 2-Methyl ketones have a strong impact on the characteristic aroma of blue cheese, contributing with fruity, floral, and musty notes [66, 67]. A comparative study of 55 *P. roqueforti* strains, using a DHS system with a Tenax® trap and analysis via GS-MS, resulted in the identification of 52 VOC. Several substances known from Camembert, like sulfur compounds (e.g., methanethiol) and alcohols (e.g., oct-1-en-3-ol), were detected. With 50–75% of the total volatiles, 2-methyl ketones were the most abundant analytes [61]. It is notable that the composition of the 2-methyl ketones in the cheese depends strongly on the stage of ripening [68], which again illustrates the growth phase-dependent VOC production.

2.2 VOC Produced During Non-food SSF of Ascomycetes

Biotransformation of industrial side streams into valuable products, such as volatile organic compounds, by means of fungal solid-state, respectively, solid-substrate fermentation, is done since ages. In most cases, filamentous fungi are used for SSF, but even yeasts have been applied on solid substrates. Rossi et al. [69] analyzed the aroma profile of the plant pathogen *Ceratocystis fimbriata* cultured in SSF on citric pulp, a residue of the citrus juice-producing industry. Citric pulp as substrate was tested solely or supplemented with soya molasses, sugarcane molasses, soya bran, or urea and different concentrations of saline solution (KH_2PO_4, $CaCl_2$, and $MgSO_4$). HS analysis was performed by means of GC-FID to identify VOC produced by the fungus. Acetaldehyde, ethanol, ethyl acetate, propyl acetate, ethyl isobutyrate, hexan-2-one, hexan-2-ol, and 3-methylbutyl acetate were identified. The addition of soya bran as N-source, sugarcane molasses as C-source, as well as saline solution to the citric pulp resulted in the highest production of total volatiles.

The amount of 3-methylbutyl acetate, a valuable banana aroma, increased 5.5-fold compared to the citrus culture without supplementation. SSF of *C. fimbriata* on cassava bagasse supplemented with either leucine or valine resulted in strong banana aroma, which was probably due to the presence of 3-methylbutyl acetate [70]. In contrast, SSF of cassava bagasse supplemented with urea showed a similar growth rate, but only a slight production of VOC. The high 3-methylbutyl acetate concentration in cultures supplemented with leucine might derive from an active Ehrlich pathway, yet not known for *C. fimbriata* but described for the ascomycete *S. cerevisiae* [71]. Here, leucine would be transformed into 3-methylbutanol, which can be converted into 3-methylbutyl acetate by an alcohol acyltransferase [72]. Contradictory to this assumption, valine would react via 2-methylpropanol into 2-methylpropyl ethanoate (isobutyl acetate) not detected by the authors [70]. On coffee residues, *C. fimbriata* showed a different VOC pattern during SSF [73]. Coffee pulp and coffee husk supplemented with glucose were used as substrates. After inoculation with spores, the volatiles in the HS were analyzed by means of GC-FID over a time period of 10 days. After 48 h, the highest amounts of volatile compounds were detected. The dominant volatiles in the HS of the coffee husk and coffee pulp samples were ethyl acetate (84.7 and 69.6%), ethanol (7.6 and 20.0%), and acetaldehyde (2.0 and 2.1%). Esters including ethyl propionate, propyl acetate, isobutyl acetate, ethyl isobutyrate, and ethyl butyrate contributed to the fruity odor of the culture, but no 3-methylbutyl acetate was detected.

In addition to the impact of substrates on the composition of VOC, the cultivation type, SSF or submerged fermentation (SmF), respectively, shaken or static cultures, has an effect on the overall productivity of VOC, e.g., for the coconut-like aroma compound 6-pentyl-α-pyrone (6-PP) in cultures of *Trichoderma* species. Kalyani et al. compared the production of 6-PP in potato extract medium supplemented with glucose in shaken and static cultures [74]. Over a period of 5 days, samples were harvested every day. Aliquots of the culture supernatant were extracted with dichloromethane, dried over sodium sulfate, concentrated, and 6-PP was quantified with GC-FID. After 96 h, the highest 6-PP concentration of 455 mg L^{-1} was obtained in the static cultures, whereas in the shaken cultures, the maximum amount of 187 mg L^{-1} was already reached after 48 h. In SSF cultures of *T. harzianum* on sugarcane bagasse, the 6-PP concentration was even higher with 933 mg L^{-1} at the end of cultivation after 10 days [75]. The productivity of 6-PP by *T. harzianum* was almost 14 times higher in SSF (171 mg L^{-1} day^{-1}) than in SmF cultures (12.5 mg L^{-1} day^{-1}) [75]. Sugarcane bagasse was also used in several other studies for 6-PP production. Araujo et al. screened 95 fungal isolates for the occurrence of 6-PP [76]. One *Trichoderma* species showed the highest product concentration with 3 mg 6-PP g^{-1} dry matter (DM) after 5 days of cultivation, accounting for approximately 940 mg 6-PP L^{-1} of supernatant [76]. Another *Trichoderma* species, *T. viride*, was cultivated on sugarcane bagasse for a period of 12 days. The highest 6-PP concentration (3.6 mg g^{-1} DM) was also achieved at day 5 [77]. It is worth mentioning that the usage of 6 g sugarcane bagasse instead of 4.5 g resulted in a significant decrease in 6-PP production (1.7 mg g^{-1} DM). Improvement of the cultivation conditions for 6-PP production by SSF of

T. harzianum on green coir powder was performed by Souza Ramos et al. [78] by altering the culture conditions regarding the composition of the supplements, the moisture content, the amount of spores for inoculation, and the temperature. After 7 days of fermentation, the HS was analyzed with SPME (PDMS) and GC-FID. The highest amount of 6-PP was achieved at 28°C by using per 100 g coir: 3 g sucrose, 0.24 g $NaNO_3$, 0.18 g $(NH_4)_2SO_4$, 0.1 g KH_2PO_4, an inoculum of 2.2×10^6 spores, and water to reach a final moisture level of 55%. The fermentation under the selected conditions led to a six times higher 6-PP production (5.0 mg g^{-1} DM) than the initial one (0.8 mg g^{-1} DM).

Another interesting fruity flavor is the pineapple odor ethyl hexanoate. Yamauchi et al. compared the production of ethyl hexanoate in SSF of *Neurospora* sp. on pregelatinized rice, wheat bran (*fusuma*), corn grits, and spent grain, supplemented with different additives [79]. Cultivation on pregelatinized rice with 5% malt broth resulted after 2 weeks in 180 mg kg^{-1} ethyl hexanoate, whereas cultures with *fusuma*, corn grits, and spent grain only reached 10 mg kg^{-1} or less. This SSF of pregelatinized rice with *Neurospora* sp. led to a *koji* used for the sake production and resulted in a fruity perception of the sake. Ethyl hexanoate is also a substantial flavor compound in traditional Chinese liquor, obtained by distilling SSF of grains fermented with *daqu*, which is a starter culture consisting of a diverse microbiome, including inter alia *Lactobacillus*, *Bacillus*, *Aspergillus*, and *Saccharomycopsis*. Zhang et al. [80] analyzed volatiles appearing during the fermentation process of *daqu* on a mixture of sorghum, corn, wheat, and rice. Cultivation was conducted at 30°C, and samples were harvested on days 1, 10, 23, 34, 48, 59, and 70. Aromatic esters were extracted with ethanol and quantified by GC-FID. Ethyl hexanoate firstly appeared on day 59 (93.3 μg g^{-1}) and only slightly increased until day 70 (96.1 μg g^{-1}). Such combinations of microorganisms can result in a diverse pattern of VOC. A mixture of orange pulp molasses, potato pulp, whey, brewer's spent grain, and malt spent rootlets was fermented by a kefir community consisting of symbiotic consortia of fungi and bacteria [81]. Volatiles in the HS of these cultures were extracted with SPME and identified using GC-MS. In the HS of kefir cultures, the highest total VOC concentrations have been determined with ε-pinene being the most abundant aroma compound (4,208 mg kg^{-1}).

2.3 VOC Produced During SSF of Basidiomycetes

Members of the phylum *Basidiomycota*, which most mushrooms belong to and referred to as basidiomycetes, have been cultivated since centuries by humans for the purpose of food production. The origin of mushroom production is in China, with the first handwritten instruction for mushroom cultivation dating back to the seventh century [82]. Generally, mushroom cultivation, which is probably the largest industrial SSF sector, is performed on agroforestry wastes, like straw, sawdust, reed grass, banana and bamboo leaves, tree bark and stems, husks, and scrubs [82–84].

In addition, side streams of the food-producing industry, like wheat straw, citrus peels, and cocoa shells, can be a valuable substrate source [85]. Worldwide, 950 different mushrooms are consumed and 50 different species are cultivated. China is the biggest commercial producer with an annual production of 7 million tons in 2013, which accounts for more than 70% of the worldwide production [86]. Several articles already reviewed the variety of aroma compounds present in fruiting bodies of fungi of the phylum Basidiomycota [87–89]. In the following, the focus is on the aroma compounds produced by the vegetative mycelium. The influence of different cultivation parameters, including substrate preparation, inoculation, and incubation time on the production of different aroma compounds, is highlighted. Moreover, also the genotype of the cultivated mushroom can have an impact on the production process and yield [90]. In addition, recent results on VOC production during the process of fructification are presented.

2.3.1 Volatiles Produced During Vegetative Growth

The fruiting bodies of the oyster mushroom *Pleurotus ostreatus* are known as a delicate food with a pleasant aroma and derive from SSF on lignocellulosic residues. When cultivated in liquid media or on an artificial solid substrate, the aroma composition of *P. ostreatus* cultures alters [91]. Kabbaj et al. compared VOC derived from *P. ostreatus* liquid cultures, agar plate cultures, and SSF on sugarcane bagasse with its fruiting bodies produced on wheat straw [91]. VOC were extracted with a DHS system equipped with a Tenax® trap. Afterward, volatiles were thermally desorbed, cryofocused, and analyzed via GC-MS. The volatile profile of the mycelium varied significantly between the different cultivation conditions. In fruiting bodies, octan-3-one contributed to 80% of the integrated peak areas, followed by octan-3-ol with about 14%. During SSF with sugarcane bagasse or on agar surface cultures, mainly octan-3-one (72.5% and 67.4%, respectively) was detected, with similar concentrations as in the fruiting bodies, whereas in liquid culture, it only contributed with 36.2% to the detected volatiles. In liquid cultures, oct-1-en-3-ol (38.5%) was the dominant compound but only found in small amounts in the SSF with sugarcane bagasse (0.3%) and agar medium (1%). It is worth mentioning that the approach with liquid medium contained relatively high quantities (16.2%) of 2-methylbutanol, resulting in a spicy and repellent note of the culture. This illustrates the impact of the culture conditions on the volatile composition. In addition to the culture conditions, the substrate itself has an effect on the VOC produced. When *P. ostreatus* was cultivated on spent leaves of *Eucalyptus cinerea* derived from the production of essential oils, the spectrum of VOC was influenced by the substrates [92]. Cultivation of *P. ostreatus* on spent *E. cinerea* leaves was carried out in propylene bags in darkness at 25°C for 30 days to completely colonize the substrate. Afterward, the bags were removed, and the substrate block was further cultivated for approximately 1 month at 20 ± 2°C and 86–90% relative humidity as well as day and night shifts to induce fructification. The volatiles of the substrate blocks after colonization and after fructification were extracted by hydrodistillation in a

Clevenger-type apparatus, a distillation method used for the extraction of essential oils. Analysis was performed with GC-FID and GC-MS. The amounts of various volatiles including eugenol, globulol, α-cadinol, longifolene, and *p*-cymene decreased during the fermentation of *P. ostreatus* on the spent leaves, whereas the quantities of 1,8-cineole and β-caryophyllene increased significantly, and sabinene hydrate appeared. The increase of the monoterpenes and β-caryophyllene might be due to an active release of these compounds by *P. ostreatus*. 1,8-Cineole was hydroxylated by the fungus to 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-ol and further oxidized to 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-one.

Wu et al. [93] analyzed the volatile biotransformation products of vine tea, a product derived from the vine species *Ampelopsis grossedentata*, during SSF with the medical mushroom *Wolfiporia cocos* (synonym: *Poria cocos*). The fungus was cultivated on moistened tea leaves at 28°C for 15 days. Unfermented vine tea inhered a strange taste, whereas fermented tea exhibited a pleasant aroma. Every third day, cultures were harvested for LLE with water and dichloromethane. The extracts were distilled for 5 h, subsequently concentrated, and analyzed by means of GC-MS. During the fermentation remarkable amounts of methyl 2-methylpentanoate were produced, resulting in the fruity note of the processed vine tea. Aroma compound production of the medically valuable mushroom *Antrodia camphorata* was observed during cultivation on millet for 25 days at 28°C [94]. On days 10, 15, 20, and 25 volatiles in the HS were analyzed by means of SPME (CAR/PDMS) and GC-MS. In total, 124 compounds were detected not present in the unfermented millet. The VOC profile varied remarkably during fermentation. In early stages, C8 compounds, such as oct-1-en-3-ol, octan-3-one, and octan-1-ol, accounted for approximately 50% of all detected VOC. In later stages, octan-3-one (32–38%) was the most abundant substance, followed by methyl 2-phenylacetate (13.1–16.6%). The relative amount of oct-1-en-3-ol decreased rapidly over the time (down to 0.9%), whereas the quantity of sesquiterpenes and lactones, including 5-butyloxolan-2-one, 5-heptyloxolan-2-one, and 6-heptyloxan-2-on, increasingly contributed to a fruit-like flavor with herbal fresh notes.

As already shown, basidiomycetes are mainly cultivated on residues from industrial or agricultural processes for human consumption. In general, the substrate used consists of lignocellulose, which can be metabolized by all commercially cultivated mushrooms using their diverse enzymatic system to a certain extent. This so-called lignocellulolytic system can also generate some volatile side products and thus might be an unexploited source for aroma compounds. The major class of lignin degrading enzymes are class II fungal peroxidases, which require H_2O_2 as a cofactor. The H_2O_2 is provided by different alcohol and glucose oxidases, such as the aryl alcohol oxidases (AAO) [95]. As a substrate, AAO demand aryl alcohols, such as benzyl alcohol, which are converted by the AAO into the almond flavor benzaldehyde [96]. Bonnarme and coworkers had a closer look into this system comparing cultivation conditions and their impact on VOC production and enzymatic activity [97, 98]. They used the white rot fungus *Bjerkandera adusta* for SSF and SmF for the production of benzaldehyde, benzyl alcohol, and benzoic

acid [97, 98]. When SmF cultures of *B. adusta* were supplemented with polyurethane foam cubes, the benzaldehyde concentration increased significantly (8.3-fold) as did the AAO activity (4.3-fold). On the other hand, the maximum benzyl alcohol concentration was higher in non-immobilized SmF cultures (1.5-fold). This emphasizes the possible usage of lignocellulolytic enzymes for the aroma biosynthesis. In a similar study, the effect of different solid supports was investigated concerning the production of aryl metabolites. In comparison to the inert carrier perlite, the utilization of lignocellulosic wheat bran for SSF of *B. adusta* resulted in a remarkable production increase of benzyl alcohol and benzaldehyde (up to tenfold). Similar to the SmF, the AAO activity was only detected in SSF on wheat bran and not in cultures where perlite was used [97].

Although the substrate can have an influence on the aroma compounds produced during SSF, the aroma profile of fruiting bodies derived from different substrates remained unchanged for the delicious black poplar mushroom *Agrocybe aegerita* [99]. *A. aegerita* was cultivated on 100% wheat straw, 100% cocoa shells, as well as wheat straw supplemented with either cocoa shells (17%), citrus pellets (17%), carrot mesh (17%), or black tea pomace (17 and 45%). Straw-based substrates showed comparable growth of *A. aegerita*, whereas on 100% cocoa shells, only marginal mycelium growth and no fruiting bodies were observed. Fruiting bodies grown on 100% wheat straw and on wheat straw supplemented with black tea pomace (45%) were compared in regard to their aroma profile. After addition of methanol and water, homogenized fruiting bodies were extracted by means of LLE. The concentrated extracts were analyzed via GC-MS/MS-O. Eleven VOC were identified in the fruiting bodies, and the volatile composition of the two different SSF cultivations did not exhibit considerable differences. C8 compounds, including oct-1-en-3-ol, oct-1-en-3-one, and octan-3-one, contributed to the typical mushroom odor, whereas 2-phenylethanol added a rose-like note to the aroma profile.

2.3.2 Volatiles Produced During Fructification

In basidiomycetes, sexual reproduction consists of a complex, multipolar mating-type system, involving a haploid, monokaryotic stage [100]. Monokaryotic mycelium of matching mating types can fuse, resulting in a dikaryotic stage. Out of this stage, fruiting bodies derive to start a new reproduction cycle. Freihorst et al. [101] compared the volatile profile during SSF of two sexual compatible monokaryotic strains of *Schizophyllum commune* with the volatile composition of a dikaryon obtained from mating both analyzed monokaryons. All three strains were cultivated on solid complex medium for 7 days at 28°C. At the end of cultivation, volatiles were extracted using HS-SPME (DVB/CAR/PDMS) for 1 h and analyzed with a GC-MS system. Methyl 2-methylbutanoate was the dominant VOC in both monokaryotic cultures (80%) and in the dikaryotic samples (31%). Apart from that, the VOC profile of monokaryons and dikaryons differed tremendously. The volatilome, the aggregate of all VOC, of the monokaryons contained

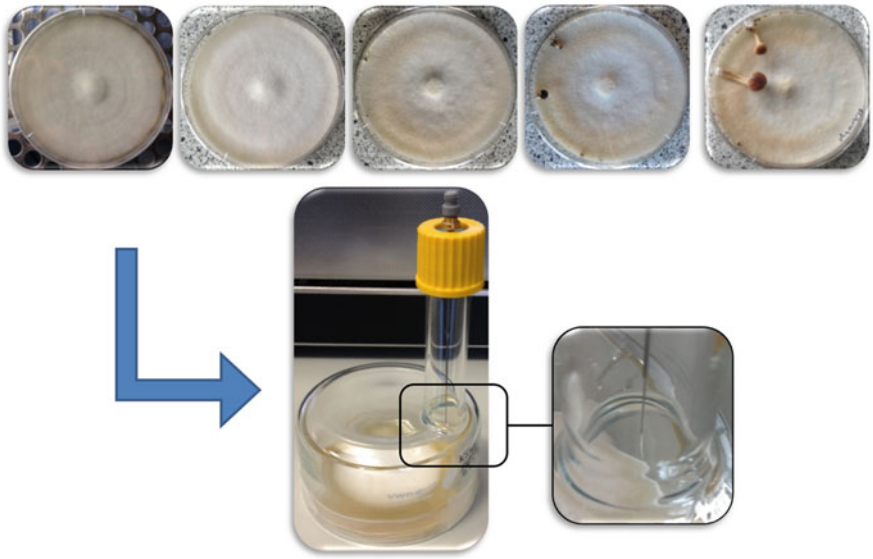


Fig. 6 Extraction of VOC with SPME during fructification of *A. aegerita* in SSF on malt extract agar using modified crystallizing dishes; photos kindly provided by Sabrina Herold

methyl 2-methylpropanoate, not found among the volatiles of the dikaryon. Vice versa, oct-1-en-3-ol, octan-3-one, *S*-methyl thioacetate, 3-methylbutan-1-ol, isobutyl acetate, and β-bisabolol were only detected in the HS of the dikaryon. Ethyl 2-methylbutanoate had a comparable ratio in the volatiles of all samples (monokaryons 1–4%, dikaryon 4%).

All these results demonstrate that cultivation phases as well as the genetic background of the analyzed specimens have a remarkable influence on the volatile composition of basidiomycetes during SSF. In a recent work of our research group (unpublished), we investigated the role of both factors on the volatilome of *A. aegerita* in SSF. Therefore, strains were cultivated on 1.5% MEA in modified crystallizing dishes (Fig. 6), allowing an efficient extraction of VOC by means of SPME. This system had the advantage to ensure aeration of the culture, necessary for the development of fruiting bodies. Furthermore, without harvesting the mushrooms, it was possible to analyze the changes of volatile composition in the HS of the same sample over the time, avoiding disturbance of the system and thus the formation of VOC artefacts due to disruption of cell compartments. Four monokaryotic strains, representing members of the "mycelium," "initials," "elongated," and "fruiter"-type, classified by Herzog et al. [102], and one dikaryotic strain were grown for 28 days.

Every second day, beginning with day 10 (agar plates were fully overgrown), volatiles were extracted in the HS using SPME (DVB/CAR/PDMS) and analyzed with GC-MS. The volatile profiles of all strains varied distinctively over the time. For the dikaryon, raised VOC concentrations were detected. In the HS of the dikaryotic

mycelium, mainly alcohols and ketones, like oct-1-en-3-ol, 2-methylbutan-1-ol, acetone, and cyclopentanone, were identified. The composition of the VOC changed significantly with the occurrence of fruiting bodies and during the sporulation phase. Here, sesquiterpenes, especially Δ^6-protoilludene, α-cubebene, and δ-cadinene, were the dominant substances. After sporulation, the amount of sesquiterpenes decreased, while additional VOC, mainly octan-3-one, appeared.

3 Conclusion

In addition to cultivation of mushrooms for food production, filamentous fungi are promising candidates for SSF applications in the aroma sector. They are able to grow on different substrates, which can result in a variable VOC profile, offering the still not fully exploited potential of gaining a whole range of valuable flavors. The diversity in these volatile profiles also depends on the developmental and reproductive stage of filamentous fungi, which facilitates the process control of SSF by observing the VOC production.

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Stimulating Production of Pigment-Type Secondary Metabolites from Soft Rotting Wood Decay Fungi ("Spalting" Fungi)

R. C. Van Court and Seri C. Robinson

Contents

Abstract A small group of soft rotting wood decay fungi produce extracellular pigments as secondary metabolites in response to stress and as a means of resource capture. These fungi are collectively known as "spalting fungi" and have been used in wood art for centuries. The pigments produced by these fungi are finding increasing usage in industrial dye applications and green energy but remain problematic to grow in batch culture. Additionally problematic is that the pigments, especially the blue-green pigment known as xylindein, produced by *Chlorociboria* species, have yet to be fully synthesized. In order to further research development of these pigments and find success in areas such as textile and paint dyeing, wood UV

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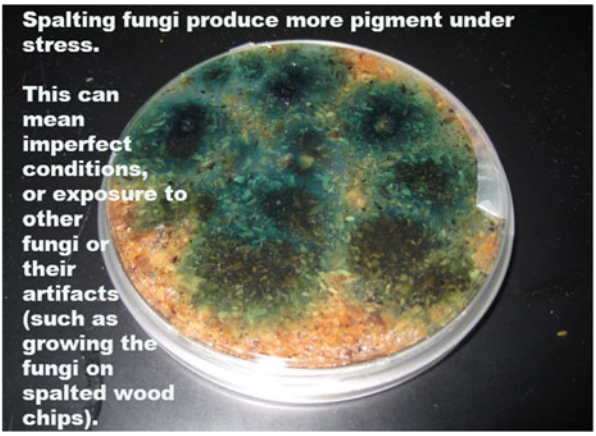
protection, and organic photovoltaic cells, methods must be developed to mass produce the pigments. To date, three distinct methods have been developed, with varying degrees of success depending upon the fungal species (amended malt agar plates, shake liquid culture, and stationary liquid culture). This chapter details these three methods, their history, advantages and disadvantages, as well as their potential for industrial scale-up in the future.

Graphical Abstract



Keywords *Chlorociboria* spp., Fungal dyes, Fungal pigments, *Scytalidium cuboideum*, *Scytalidium ganodermophthorum*, Secondary metabolites, Spalting, Zone lines

1 Part I. Background

1.1 Fungal Pigments: Useful Secondary Metabolites

Fungal species produce a range of pigments, with colors spanning the full spectrum of the rainbow. These organic compounds include carotenoids, melanins, flavins, phenazines, quinones, monascins, violacein, and indigo [1]. Production of pigments is widespread across fungal species and lifestyles, ranging from a red produced by insect-pathogenic *Cordyceps* variety [2] to blues from terrestrial *Crocinoboletus* spp. [3, 4].

Fungal pigments (always referred to as "pigments," even if they act more like dyes) are considered secondary metabolites – low molecular weight compounds not directly necessary for growth and development. Secondary metabolites are generally

understood to confer on the organism a competitive advantage, increasing their adaption to ecological niches [5]. This has been demonstrated by the work of Rohlfs et al. [6], who showed that loss of secondary metabolites by mutation leads to decreased resistance to fungivory of *Aspergillus nidulans* (Eidam) G. Winter compared to wild type. The activity of many of these fungal compounds has led to their utility in industry, such as production of antibiotics, immunosuppressants, and statins [7, 8].

While the specific roles performed by many pigments are still unknown, those roles that have been identified are exceptionally diverse. Carotenoids, which are a range of colors from yellow to red, have a protective role against oxidative stress and UV and visible light, in addition to being used as intermediaries in compound synthesis [9]. Other pigments are antagonistic. The red-colored cercosporin produced by *Cercospora* spp. is a particularly interesting example, playing a role in the pathogenicity of the organism. A photosensitizer, when exposed to bright light, generates active oxygen species leading to rapid damage of the membranes of plant cells it infects, and its broad-spectrum toxicity has also been demonstrated against fungi, mice, and bacteria [10]. Fungal pigments produced by *Monascus ruber* have also been shown to have antimicrobial activity against bacteria, with different pigments providing varying inhibitory effects [11].

The activity of fungal pigments has led to their use in a variety of industrial applications, most prominently in the food industry and medical fields. In the food industry, pigments have been used as food colorants. *Monascus* species in particular have been traditionally used in Asia to produce red colors and are being further refined for use as industrial food colorants [12–16]. In the medical field, fungal pigments are under investigation for use as anticancer and antimicrobial agents, antioxidants, and bioindicators [17].

These species used for pigment production on industrial scale are generally saprobic fungi, deriving nutrients from dead organic matter. This has allowed for straightforward pigment production using liquid cultures, which is industry standard. However, there is a small group of wood decay fungi, known as soft rots, some of which release very specific types of pigments into wood and are not easily grown in liquid batch cultures. These naphthoquinone pigments have unusual UV stability, hydrophobicity, and color stability, making them ideal not just for historic wood coloration but also for textile dyes, paint colorants, decking protection, and organic photovoltaics. The remaining hurdle for industrial use of these pigments is production scale-up, as most have very specific growth requirements that must be met in order to produce significant quantities of their pigments.

1.2 Spalting Fungi

Spalting is the colorization of wood by fungi, produced as a part of the normal life cycle of certain species of decay fungi [18]. This can be due to either the breakdown

of colored compounds in the wood or through their production. There are three main kinds of spalting: bleaching, zone line formation, and pigmentation.

Bleaching is caused by the breakdown of colored lignin from the wood cell wall, generally by fungi classified as white-rotting, which results in a lightening of the natural wood color [19, 20]. Heavy colonization by these fungi has a profound effect on the structural integrity of the wood, making it softer and weaker. A lightening in color can also be due to a buildup of white mycelium [21].

Zone line formation is characterized by production of darkly pigmented winding lines in wood that are made up of melanized hyphae [22]. Zone lines are formed in response to changes of moisture content in wood [23] and atmospheric conditions such as presence of increased CO_2 [24] and as a barrage reaction caused by antagonism between dikaryons [25, 26]. Most fungi that produce zone lines are basidiomycetes, though ascomycetes such as *Xylaria polymorpha* (Pers.) Grev. are also known to do so [27]. Microscopic investigation of zone line formation across selected species and wood types has shown two main forms of pigment deposition: a melanin granule dense layer formed on the lumen of wood cell walls and a dense packing of sclerotial hyphae fully obstructing the lumina [28, 29]. The sealing off of lumina by fungi is considered a protective response to stress, be it from competition or environmental change.

Pigmenting fungi are the final form of spalting fungi and produce internal coloration of wood. Most pigmenting fungi are ascomycetes, from which basidiomycetes are considered to have evolved. The most common wood pigmentation is "blue stain," caused by exposure to airborne, soilborne, or beetle-borne fungi, and is considered a defect in the lumber industry [30]. However, blue stain is not the only form of pigmentation of wood; other vibrant colors such as red produced by *Scytalidium cuboideum* (Sacc. & Ellis) Sigler & Kang, yellow produced by *Scytalidium ganodermophthorum* Kang, Sigler, Lee & Yun, and blue-green produced by *Chlorociboria* spp. can also be seen.

Spalted wood has a history of use in fine wood artwork [18]. *Chlorociboria* spp. blue-green stained wood has been used since the fifteenth century in intarsia, a form of inlay, where the blue-green color was used for natural scenery to replicate the color of leaves, for simulation of fabric, and to represent stone [31]. More recently, zone line spalted wood was introduced to the woodturning community in the 1970s by the Lindquist [32] and has become an exceptionally popular material to work with. An example of a contemporary piece of wood turning showing both zone lines and pigmentation can be seen in Fig. 1. The popularity and the rarity of pieces on the market drive the high economic value placed upon spalted wood, making its production not only an exercise in engendering natural beauty but also increasing financial profit.

Fig. 1 Contemporary spalted wood art. Turned bowl with induced spalting by Dr. Seri Robinson, with forms of spalting including blue, orange, pink, purple, and yellow pigmentation in addition to white rot and zone lines. Image copyright northernspalting.com

1.3 Soft Rotting Fungi and Their Pigments

Pigmenting spalting fungal species are considered soft rotting ascomycetes, producing a distinctive form of decay in wood. The name "soft rot" was applied by Savory [33] to characterize a generally superficial form of decay associated with wood discoloration and softening, in which cellulosic breakdown gives the wood a cracked appearance when dried. Soft rot fungi are commonly found in conditions not effectively colonized by more aggressive wood decay fungi, like basidiomycetes, such as in exceptionally high moisture environments like water towers [34]. They also show varying attack patterns, with carbohydrates degraded at different rates depending on wood species [35].

Current research focuses on four species of pigmenting spalting fungi in the order *Helotiales* for industrial applications. Two of these, *Chlorociboria aeruginosa* (Oeder) Seaver ex C.S. Ramamurthi, Korf & L.R. Batra and *Chlorociboria aeruginascens* (Nyl.) Kanouse ex Ram., Korf & Bat., are North American species that both produce the blue-green pigment xylindein distinctive of the genus [36, 37]. *Scytalidium cuboideum* (Sacc and Ellis) Singler and Kang produces the red/pink pigment dramada in wood, which is a naphthoquinonic pigment (colloquially known as draconian red) and also a naturally occurring crystal [38]. Finally,

Scytalidium ganodermophthorum Kang, Sigler, Lee & Yun produces an as yet undescribed yellow pigment. The extracted pigments have been physiologically characterized by Vega Gutierrez and Robinson [39]. Scanning electron microscopy (SEM) showed that the pigment from *S. cuboideum* created filament-like structures that wrapped around substrates such as fibers to which it was applied, forming flowerlike amalgamations. Pigment from *C. aeruginosa* was found to form a relatively smooth amorphous film and *S. ganodermophthorum* a bumpy and uneven surface.

Despite the structure of the pigment of *S. ganodermophthorum* being unknown, its role in the ecology of the fungus is the best understood of all studied pigmenting spalting fungi, indicated as playing a role in the pathogenicity of the fungus. This species was identified as the causal agent behind yellow rot of reishi (*Ganoderma lucidum* (Curtis) P. Karst), a popular cultivated medicinal, by Kang et al. [40] in Korea. The authors theorized that the yellow pigment aided in pathogenicity, commenting that culture isolates triggered lysis of *G. lucidum* cell membranes and referencing a previous study by Oh et al. [41] where the pigment inhibited *G. lucidum* growth. However, the fungus is also associated with wood decay and produces pigment in wood like other species in the genus, fully completing its life cycle without infection [40]. This suggests that while the pigment has utility as a pathogenic agent, this likely developed from pigment already in production that may confer an additional adaptive advantage.

The role of the pigment of *S. cuboideum*, while in the same genus, is less understood. *Scytalidium cuboideum* has been isolated from a variety of pink stained lumber and has even been found to grow on red oak (*Quercus rubra* L.) treated with sodium pentachlorophenol [42]. When grown with other fungi, *S. cuboideum* is often less competitive despite being a fast grower alone, occasionally producing pink zone lines that are likely a competitive response analogous to melanized zone lines. The pigment may also be UV and light protective [43–46]. Interestingly, *S. cuboideum* has been isolated from clinical samples submitted to the University of Texas Health Science Center, though did not produce pink pigmentation on PDA as would be expected of the fungi [47]. This may suggest that the pigment confers a particular competitive advantage in plant-based substrate.

The blue-green pigment xylindein produced by *Chlorociboria* sp. is conserved throughout the genus, suggesting it confers a powerful ecological advantage for the fungi though there has been no conclusive evidence as to what it may be. One line of thought is that the pigment may have a powerfully protective effect against the environment, due to its incredible stability. Blue-green pigmented wood from the fifteenth century retains coloration today [31], and recent experimentation has found xylindein to be exceptionally photostable and heat stable, with film properties unaffected up to 180°C and degradation occurring at temperatures as high as 210°C [48]. As a coating and exposed to a weathering chamber, xylindein showed potential to protect wood from degradation [49].

Xylindein may also protect the fungi from other competitors. A 1913 patent reported in Robinson et al. [18] was filed for use of the pigment as an antimicrobial agent, and a more recent 2007 patent was filed for use as plant germination inhibitor

[50]. These suggest bioactivity. Interestingly, this use was described as more effective after exposure to strong UV light, which turned the pigment purple.

It is hypothesized that the production of these pigments is a form of resource capture by the fungi based on variations in fungal growth as exemplified by *Chlorociboria* spp. *Chlorociboria* species are found on heavily decayed wood though do not cause significant decay suggesting they are late-stage colonizers. With other fungi already in the wood, use of pigment to demarcate territory, similar to the more focused deposition of melanin in zone lines, may be an effective competitive strategy. Production of xylindein as a competitive response is suggested by the loss of pigment production in nutrient-rich media over time that has been seen in multiple publications [51, 52].

Pigment production as a response to competition is also hinted at by variable responses to growth on white-rotted and sound wood. *Chlorociboria* sp. growth on wood pre-treated with a white-rotting fungus showed a possible, but nonsignificant, stimulatory growth effect for some species when compared to untreated wood [53–55]. Spalted aspen also showed somewhat faster *Chlorociboria* growth compared to unspalted aspen when added to malt agar plates, though with time this effect was no longer seen [56–58]. The increased sugars available in decayed wood likely influence the increase in growth rate; however it is also possible that the evidence of other fungi in the wood may stimulate xylindein production.

If the pigment is produced as a competitive or stress response, it is speculated that antagonizing the fungi results in more pigment production, as the stressed fungi attempt to "control" their environment. Working with this theory, addition of spalted wood chips to solid media, variations in moisture and growth substrate, and agitation of liquid media have been explored as methods to increase pigment yield. Increase in yield is critical because of the relative rarity and slow growth of these fungi, especially *Chlorociboria* spp., which makes garnering enough pigment to study a time-consuming and laborious process. In order for there to be a chance for industrial adoption, identification of a growth method that stimulates industrial-scale pigment production is necessary.

1.4 Inducing Pigment Production: Wood Species

Initial investigation of spalting conditions focused on identification of fungal interactions and wood species that could produce superior pigmentation. Numerous studies have found sugar maple (*Acer saccharum* Marsh.) to be an ideal substrate for the production of zone lines [43–46, 53–60]. For pigmenting fungi, research by Robinson and Laks (2010) found that a *Chlorociboria* sp. showed increased pigmentation and growth on trembling aspen (*Populus tremuloides* Michx.) compared to other tested species. *Scytalidium cuboideum* has been found to effectively colonize a range of species, and though a strong competitor against other spalting fungi, in wood block cultures, inoculation onto sterile media was required [61–63].

1.5 Inducing Pigment Production: Incubation Conditions

Ideal conditions for stimulating pigment production from spalting fungi differ from those required to stimulate fungal growth. Replacing vermiculate for soil in standard growth chambers was shown to improve pigmentation for tested fungal species on sugar maple [64], and that placement of the wood block above rather than below the level of vermiculite increased pigmentation for some fungi [61–63]. Further research has also shown that copper compounds such as copper sulfate can be used to produce exclusion areas [53–55] and stimulate spalting [61–63].

Ideal moisture content has also been investigated, with results variable by species. Zone line-forming species such as *Trametes versicolor* and *Xylaria polymorpha* were found to have increasing pigmentation at low initial moisture contents (29–33% in sugar maple and 29–32% in beech (*Fagus grandifolia* L.), though other zone line species such as *Polyporus brumalis* (Pers.) Fr. showed increased pigmentation at high moisture contents (59–96% in sugar maple and 26–41% in beech) [65].

1.6 The Challenges

While spalting fungi can be reliably grown on wood for decorative purposes, it is much more difficult to grow them in culture specifically to collect and extract their pigment. *Chlorociboria* spp. have been especially challenging. In addition to being extremely slow growers, this genus often ceases production of xylindein when in laboratory storage [51, 52], and its complexity has made it difficult to synthesize. Work on xylindein synthesis through dimerization of lactone produced a 7,9-dideoxy analogue, though did not progress further [66, 67]. Later work by Donner et al. [68] took a different approach and described a methodology to produce a pyranonaphthoquinone corresponding to half of the xylindein framework. The difficulty of synthesis has led to research specifically focused on generating pigment production (*not* fungal growth) in batch culture specifically for spalting fungi and investigations into the unique environments required to stimulate pigment production.

2 Part II. Batch Culture: Amended Malt Agar Plates

Malt agar plates amended with wood chips have been found to be the superior growing media for pigmenting spalting fungi. *Chlorociboria* spp. have historically been particularly difficult to grow in culture, with 2 percent malt agar plate colonies failing to fully pigment or colonize plates even after 8 months of growth [53–55]. In order to improve growth in plate cultures, wood chips of different species were

added to malt media. The addition of *Acer saccharum* yielded increased growth and pigmentation across multiple *Chlorociboria aeruginascens* isolates [56–58]. This result was supported by Tudor et al. [28, 29], who additionally found that cultures from apothecia and stroma resulted in similar growth responses across a variety of solid media.

Wood chip-amended plates have also been found to be effective growing media for *S. cuboideum* and *S. ganodermophthorum*. These fungi are much faster growing than the *Chlorociboria* cultures and can fairly quickly produce a large amount of pigment. However, due to the unique color changes of their pigments, cultures must be closely monitored and harvested when at the desired color. It has been seen in multiple publications that the red pigment produced by *Scytalidium cuboideum* becomes blue under certain conditions. This was noted when first isolated by Chidester [69] and was in wood block cultures older than 8 weeks [61–63]. This was partially explained by Golinski et al. [70], who showed that when the pigment was dissolved in basic or high polarity solvents, it turned blue. Research into the effects of the pH of media found that the highest intensity blue coloration was seen on wood samples with a pH of 8 and a superior red color at a pH of 6 [71]. *Scytalidium ganodermophthorum* also demonstrates changes in pigmentation, with plates starting a bright yellow color after inoculation and then becoming a deep red-brown color with age. When extracted, the color of the pigment in solution can range from yellow to olive green to deep red to purple. Investigations into exactly why this occurs are ongoing, though the effect is likely polarity or pH driven as for *S. cuboideum* and may also be related to concentration.

The use of wood chip-amended plates has also allowed for a new method of extraction. After complete pigmentation of plates by fungal growth, the media can be dried and blended to a powder from which pigments can be extracted using a selected solvent [43–46]. The blending followed by extraction method cannot be used on traditional malt agar plates, as the media will partially dissolve along with the pigment. DCM has been identified as the most effective solvent fungal pigments, giving a fast extraction from growth media and good solubilization of dried extracted pigment [43–46].

3 Part III. Batch Culture: Liquid Stationary Culture

Spalting pigmenting fungi under investigation have been found to grow effectively in liquid 2 percent malt media, although exhibit varying growth characteristics as described by [72]. *Chlorociboria* spp. were found to be slow growing, with mycelium forming discreet fluffy clumps and the minimal xylindein production aggregating and adhering to the glass of culture flasks. Recent investigations have since not seen any evidence of extracellular pigment production by *Chlorociboria* sp. in liquid cultures. However, it was noted other strains of *Chlorociboria* have been observed to grow as mycelial mats near the top of liquid culture media. Both

S. cuboideum and *S. ganodermophthorum* form mycelial mats when stationary, with pigments not adhering to the glass but staying in solution.

Extraction of pigment from liquid cultures consists of blending hyphae and media with a small electric blender, combining this with DCM, shaking, and using a separatory funnel to collect the pigmented DCM layer. Blending of solution before extraction yielded visibly more pigment compared to just extracting the water-solubilized pigment [72]. Blending is particularly important to extract pigment from *Chlorociboria* spp. and to some extent for *S. ganodermophthorum* which had increased hyphal pigmentation when compared to *S. cuboideum*.

Static cultures seem to allow for increased growth for *Chlorociboria* spp. over shake cultures, making them preferred for xylindein production. Blending of mature *Chlorociboria* cultures produces such concentration of pigment that the dark green color appears almost black. Further investigations into variations of liquid medias used for *Chlorociboria* growth are ongoing.

4 Part IV. Batch Culture: Liquid Shake Culture

Shaking has been used as a method to stimulate pigment development, working under the assumption that this stresses the fungi and invokes pigment production. Shake culture was identified as the most effective form of liquid media growth by [72], citing faster media pigmentation when compared to static cultures. *Chlorociboria* spp. in shake culture at 110 rpm were shown to grow in clumps similar to static cultures, though there was less adherence of xylindein to the culture glassware and hyphal mats took longer to form. *Scytalidium cuboideum* and *S. ganodermophthorum* in the same conditions grew dispersed throughout the volume of the flasks and showed good production of pigment. *Scytalidium cuboideum* showed fast pigmentation of media in 2 to 3 days, with an initial pink-red coloration that became deep red with time. Interestingly, pigmentation declined after day 3, not increasing again until between 16 and 48 days. *Scytalidium ganodermophthorum* cultures showed pigmentation of media in 2 to 3 days like *S. cuboideum*, with coloration quickly increasing until day 8 and then slowing. The color of the culture changed with time, starting at light yellow and moving to a yellow/red/brown coloration with increased pigment concentration similar to that seen in solid media.

These results suggest that shake culture is the most effective liquid media technique for the tested *Scytalidium*s, especially *S. cuboideum*, as extracellular pigment is produced and can be harvested directly. This allows for relative ease of maintaining culture and the possibility of continuous fermentation, though shake cultures do seem more prone to contamination and sterility of glassware is critical. *Chlorociboria* spp. in liquid culture do pigment the media with time; however this seems to be a result of hyphae breaking causing pigment release instead of pigment secretion. When liquid cultures are filtered, collected hyphae are highly pigmented and liquid only faintly so.

5 Part V. Applications and Future Developments of Spalting Technology

Spalted wood, especially zone line spalted wood, is a highly priced wood product. Spalting underutilized or low-value woods therefore increases their market value and generates profit for the producer. Application of fungal pigments has been found to mitigate the visual effects of blue stain in pine, normally a product associated with economic loss [73], and effective coloration of bamboo has been demonstrated [74]. On a commercial scale, large non-sterile logs have also been found to be an effective spalting substrate, producing good zone lines and stain [75].

Direct use of pigment carried in DCM as a colorant has been compared to fungal-induced spalting, and for most of the 16 species of wood tested, the use of pigments from *C. aeruginosa*, *S. cuboideum*, and *S. ganodermophthorum* carried in DCM yielded higher pigmentation than direct inoculation [43–46]. These extracted pigments in DCM and liquid media have also been favorably compared to aniline dyes as wood colorants [43–46].

DCM is the most effective solvent for solubilization of the pigments, due to their limited solubility, but is a harsh chemical which makes applications more difficult. This limited solubility complicates methods of application, in addition to making characterization of the pigments and investigations into their purity more difficult. Because of this solvents and carriers beyond DCM have also been investigated. Acetonitrile worked moderately well at solubilizing pigments from *C. aeruginosa* and *S. cuboideum* [76], and natural oils, like linseed oil, have also proven effective [77]. Investigations into making artist paints using linseed oil are also ongoing, but results indicate that the pigments react to the oil over time and decolor [78]. The extracted red pigment when used as a stain on wood has also been found to be stable in response to natural and artificial UV lights [56–58] and is under investigation as a coating for decking.

The use of extracted fungal pigment has opened up a variety of applications beyond colorizing wood, and one of the most promising is the dying of textiles. The current textile dying industry is associated with significant environmental impacts, and interest is growing in more sustainable and less toxic natural pigments. Extracted pigments from *Scytalidium cuboideum*, *Scytalidium ganodermophthorum*, and *Chlorociboria aeruginosa* have been found to have strong potential as fabric dyes [79], with xylindein and dramada showing good colorfastness without mordants [80, 81] and in some cases outperforming commercial dyes [82].

In addition to use as coloring agents, one of the most exciting potential applications of spalting fungal pigments is their use in organic photovoltaic cells and other (opto)electronics. Photovoltaic technology based on organic semiconductors has great potential as a low-cost, highly flexible, and renewable technology, though at present has low efficiency [83] making development of new materials important. While pigments from *Scytalidium cuboideum* and *Scytalidium ganodermophthorum* were found to have poor semiconductive capability, the blue-green pigment from *Chlorociboria* has been found to have promising optoelectronic properties and

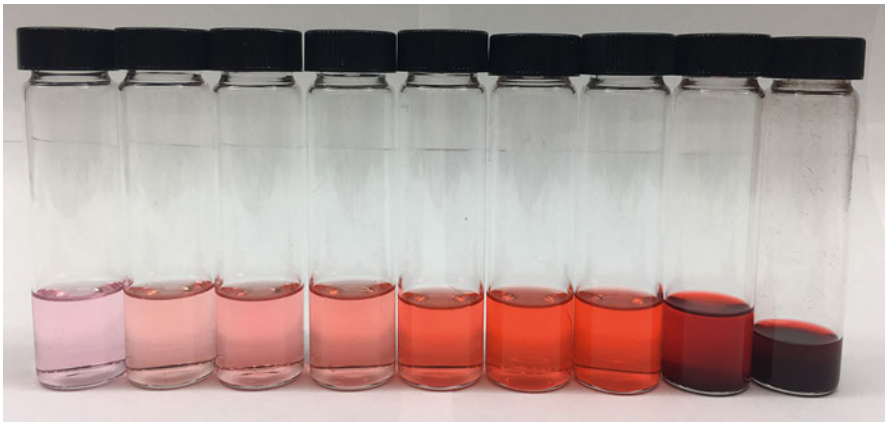


Fig. 2 Variety of colors produced by increasing concentrations of red pigment from *S. cuboideum* in acetone, ranging from 0.024 mM (left) to 19 mM (right). Image copyright R. C. Van Court

impressive stability [48, 84] and is under development as a component in organic photovoltaics.

In addition to these applications, recent development of a methodology to produce a solid form of the pigments should allow for new developments and further refinement of existing technologies. When the DCM of pigmented solutions is evaporated off, the pigments bind to the glass of their containers instead of forming a collectable particulate. This has prevented further commercialization, as the amount of pigment required to produce color change could not be measured. However, recently a method to crystalize the red pigment from *S. cuboideum* has been developed [38] which has allowed for an association to be developed between the molarity of the solvent and its color [85]. Very small amounts of pigment produced a variety of colors ranging from deep red to bright orange to light pink, suggesting final coloration of products can be fine-tuned and economically favorable (Fig. 2). The production of a solid, pure form of one spalting pigment suggests that this will also be possible for the other pigments, allowing for easier product development and study.

The ability to relate concentration to produced color will also allow for more specific studies on the toxicity of the pigments. As none of the pigments are water-soluble (with very slight solubility by *S. cuboideum* being the exception), when applied to wood or other substrates, they are not likely to break down and cause environmental concerns even if somewhat toxic. Toxicology studies may also further inform about the activity of the pigments and their ecological roles.

The final and most pressing challenge facing spalting fungi is overcoming their limited speed of fungal growth. Despite research into liquid fermentation, thus far solid-state fermentation methods of production are preferred for ease and efficiency, mirroring other studies that have found pigment production to be the most efficient in solid state [86, 87]. Future research into media variations and diving into the world of genetic analysis and manipulation will increase production of these pigments and drive future adoption of new technologies.

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Fermented Solids and Their Application in the Production of Organic Compounds of Biotechnological Interest

Nadia Krieger, Glauco Silva Dias, Robson Carlos Alnoch, and David Alexander Mitchell

Contents

Abstract We review the application of dry fermented solids (DFS) containing naturally immobilized enzymes as catalysts in synthesis and in hydrolysis reactions. The most studied application is the use of DFS containing lipases in the synthesis of biodiesel esters, by transesterification of oils or by esterification of fatty acids with short-chain alcohols in solvent-free reaction media. Other applications of DFS that have been studied include the use of DFS containing

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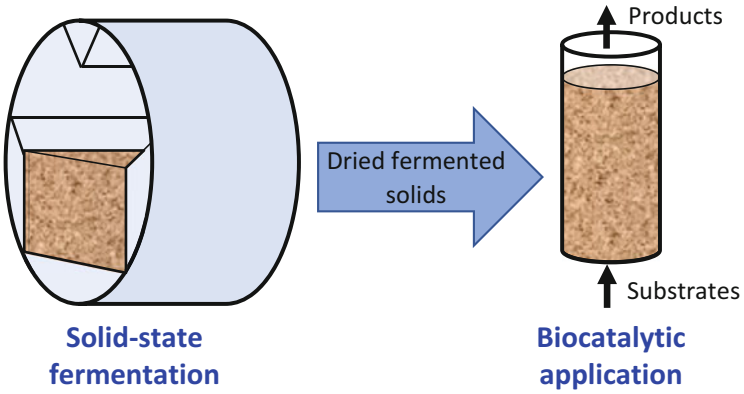
pectinases to liberate D-galacturonic acid from pectin and the production of high-value compounds by DFS containing lipases, such as the synthesis of sugar esters and the production of pure enantiomers by resolution of racemic mixtures. To date, studies are limited to proof of concept, and there are still many challenges to be faced in the development of industrial-scale processes using DFS as catalysts. A key challenge is the relatively low activity of DFS compared to commercial enzyme preparations. Attention needs to be given to scale up, not only of the bioreactor for the application of the DFS but also for the production of the fermented solids. There is also a need for economic feasibility studies to determine whether the production of DFS and their use as catalysts can be competitive at industrial scale.

Graphical Abstract



Keywords Biodiesel, Dry fermented solid, Esterification, Solid-state fermentation, Transesterification

1 Introduction

At the time of harvesting of a solid-state fermentation (SSF) process, the solid medium contains residual substrate, microbial biomass, and microbial metabolites. This medium has been referred to as "fermented solids." The chemical composition and the physical characteristics of these fermented solids can be quite different from those of the original substrate. This is not only due to the presence of the microbial biomass but also due to the degradation of substrate components during the growth of the microorganism.

Among the metabolites produced in SSF, microbial enzymes are of key interest. Many processes for the production of enzyme preparations using SSF involve several steps, including the fermentation process in which the enzymes are produced by the microorganism and subsequent processing steps in which the enzymes are

extracted and then purified, if a purified enzyme is necessary. In the final application of the enzymes, they are often immobilized. The advantage of using enzymes in immobilized form is that, in batch reactors, they can easily be removed from the reaction medium and reused in a subsequent batch; in continuous bioreactors, they can be confined within the bioreactor, allowing catalysis of the reaction over long periods. Since an immobilized enzyme can catalyze the conversion of a large amount of substrate, the contribution of the enzyme to the overall process costs is potentially lower than if a free enzyme were used: a free enzyme would be limited to catalyzing the reaction in a single batch, since it is not feasible to recover it after the reaction. However, the potential cost savings gained by using immobilized enzymes must be balanced against the costs of the immobilization procedure.

Faced with the costs of the classical route for producing immobilized enzymes for biocatalytic processes, we had the idea of producing the enzyme by solid-state fermentation, but not extracting it. Rather, the fermented solids would be dried and would act as a natural immobilization support for the enzyme. It should be noted that this is only feasible in those applications in which it is possible to use crude enzyme preparations, as all enzymes produced by the microorganism are present. Also, for this idea to be practical, the enzymatic activity contained within the DFS must be sufficiently stable over repeated batches and typically should not leach out into the reaction medium.

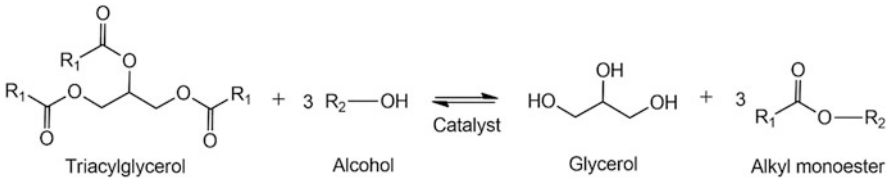
In our initial studies, we used lipases produced on corn bran to catalyze esterification and transesterification reactions in organic media [1]. While these studies were in progress, the paper of Nagy et al. [2] appeared; these authors used fermented solids containing lipases to catalyze the resolution of racemic mixtures of secondary alcohols. In this review, we describe the applications that have been developed since then, by our group and by other groups, involving the use of dry fermented solids as biocatalysts in processes for the production of organic compounds. We will start with the application of dry fermented solids in biodiesel synthesis, given that this is the most studied application.

In this review, we will refer to "dry fermented solids containing enzymes" simply as "fermented solids" and use the acronym DFS. Also, enzyme activities of the DFS are presented in U g^{-1} (units per gram of DFS).

2 Use of Dry Fermented Solids in Biodiesel Synthesis

2.1 Fermented Solids as a Cheaper Source of Lipases for Enzymatic Biodiesel

Biodiesel is a mixture of esters that is normally produced by the transesterification of vegetable oils with short-chain alcohols. The most commonly used alcohol is methanol, due to its high reactivity, although ethanol is less toxic and can be produced from renewable resources by fermentation (Scheme 1). The chemical



Scheme 1 Transesterification of a triacylglycerol with an alcohol, producing glycerol and biodiesel esters

route for biodiesel synthesis uses a strong base as catalyst, such as KOH or NaOH. It gives a high productivity, with the conversion being complete in less than 1 h. However, the chemical route has several drawbacks. First, it is necessary to use relatively high-quality vegetable oils, with free fatty acid contents below 3%; otherwise the free fatty acids form soaps with the catalyst, with these soaps making purification of the biodiesel more difficult and leading to a more impure glycerol by-product, decreasing its value. Second, even with high-quality feedstocks, it is necessary to remove the catalyst after the process, and this requires relatively large volumes of wash water [3, 4].

The enzymatic route using lipases (EC 3.1.1.3) is a more environmentally friendly alternative for biodiesel synthesis. The biodiesel and the glycerol are produced in purer forms, and the generation of waste wash water is avoided [5]. However, the enzymatic process also has significant disadvantages in relation to the chemical process: it has a lower productivity, and the costs of the enzyme are quite high. It is, therefore, essential to decrease the costs of the enzyme. Enzyme costs can be decreased by finding new enzymes that have high activity and stability in organic media, allowing their reuse over repeated cycles. In order to be reused, the enzymes must be immobilized. As mentioned above, traditional processes for immobilizing enzymes involve the steps of extraction, concentration, and immobilization. The use of fermented solids potentially allows a cheaper way of producing lipases for use as biocatalysts for biodiesel synthesis.

Motivated by the potential to reduce costs of biodiesel production, our group and other groups have studied the use of DFS containing lipases for the production of biodiesel in transesterification and esterification reactions (Table 1) [1, 6–20]. Although studies were initially undertaken in the presence of cosolvents, such as *n*-hexane or *n*-heptane, more recent efforts have focused on the use of solvent-free systems, in which the initial reaction medium contains only the substrates (short-chain alcohols and either oils or fatty acid mixtures). Solvent-free systems have the potential to reduce costs further by allowing higher volumetric productivities to be obtained and by eliminating the need to separate solvents from the final reaction mixture and recycle them. The major challenge with solvent-free systems is to overcome the deleterious effect of short-chain alcohols on the enzyme. In solvent-free media, these alcohols can strip the water molecules from the aqueous shell around the lipase, leading to denaturation. Additionally, the high alcohol concentrations can lead to a significant degree of competitive inhibition of the lipase. Further details are discussed in the following subsections.

Table 1 Studies of the direct application of dry fermented solids for biodiesel production

Type of reaction	Substrate used to produce the dry fermented solid	Microorganism	Alcohol and acid/oil (molar ratio)	Solvent	Bioreactor	Conversion in time taken	Reference
Esterification	Corn bran and corn oil	*Burkholderia contaminans*	Ethanol and oleic acid (5:1)	*n*-heptane	Shake flasks	94% in 18 h	[1]
	Perlite and nutrient solution	*Rhizopus* sp.	Ethanol and oleic acid (5:1)	*n*-hexane	10-mL stirred reactor	98% in 1 h	[6]
	Sugarcane bagasse and sunflower seed meal	*Burkholderia contaminans*	Ethanol and fatty acids from soybean soapstock acid oil (3:1)	Solvent-free	Packed bed	92% in 31 h	[7]
	Babassu cake	*Rhizomucor miehei*	Ethanol and *Acrocomia aculeata* acid oil (1:1)	Solvent-free	Shake flasks	91% in 8 h	[8]
	Wheat bran, sugarcane bagasse, and urea	*Rhizopus microsporus*	Ethanol and oleic acid (1.5:1)	Solvent-free	Shake flasks	69% in 48 h	[9]
	Sugarcane bagasse and sunflower seed meal	*Burkholderia contaminans*	Ethanol and olein (1.5:1)	Solvent-free	Packed bed	88% in 24 h	[10]
	Babassu cake	*Rhizomucor miehei*	Ethanol and palm and soybean fatty acid distillates (1:1)	Solvent-free	Shake flasks	67–74% in 6 h	[11]
	Babassu cake	*Rhizomucor miehei*	Ethanol and *Acrocomia aculeata* acid oil (6:1)	Solvent-free	Shake flasks	85% in 96 h	[12]
	Sugarcane bagasse and nutrient solution	*Rhizopus microsporus*	Ethanol and oleic acid (10:1)	Solvent-free	Shake flasks	98% in 48 h	[13]
	Cottonseed meal	*Rhizomucor miehei*	Ethanol or methanol and oleic acid (2:1)	Solvent-free	Shake flasks	85% in 4 h	[14]
Transesterification	Corn bran and corn oil	*Burkholderia contaminans*	Ethanol and corn oil (5:1)	*n*-heptane	Shake flasks	95% in 120 h	[1]
	Sugarcane bagasse and sunflower seed meal	*Burkholderia contaminans*	Ethanol and soybean oil (3:1)	Solvent-free	Packed bed	95% in 46 h	[15]
	Sugarcane bagasse and sunflower seed meal	*Burkholderia cenocepacia*	Ethanol and soybean oil (4:1)	*tert*-butanol	Shake flasks	86% in 96 h	[16]

(continued)

Table 1 (continued)

Type of reaction	Substrate used to produce the dry fermented solid	Microorganism	Alcohol and acid/oil (molar ratio)	Solvent	Bioreactor	Conversion in time taken	Reference
	Sugarcane bagasse and sunflower seed meal	*Burkholderia cenocepacia*	Ethanol and soybean oil (4.3:1)	*tert*-butanol	Shake flasks	91% in 96 h	[17]
	Sugarcane bagasse and nutrient solution	*Rhizopus microsporus*	Ethanol and corn oil (3:1)	Solvent-free	Shake flasks	68% in 72 h	[18]
	Babassu cake	*Rhizomucor miehei*	Butanol and *Acrocomia aculeata* oil (5.47:1)	Solvent-free	Shake flasks	80% in 70 h	[19]
	Sugarcane bagasse and soybean oil	*Burkholderia contaminans*	Ethanol and palm oil (5:1)	Solvent-free	Packed bed	89% in 30 h	[20]
	Cottonseed meal	*Rhizomucor miehei*	Ethanol or methanol and *Acrocomia aculeata* oil (2:1)	Solvent-free	Shake flasks	76% in 24 h	[14]

2.2 Proof of Concept: Production of Biodiesel Esters Using Dry Fermented Solids

In the early studies of our group about the use of fermented solids, we used a bacterial strain (LTEB11) that was originally identified as *Burkholderia cepacia* but which was recently reclassified as *B. contaminans*, based on genomic analysis (unpublished data). Hereafter, it will be referred to as *B. contaminans* LTEB11, even in those cases in which the original work referred to it as *B. cepacia* LTEB11. This strain produces a lipase when cultivated in submerged fermentation. After immobilization on Accurel (a commercial preparation of microscopic polypropylene beads which are hydrophobic and have a high specific surface area), this lipase is quite active and stable in organic media, catalyzing ester synthesis with yields comparable to those that have been reported using commercial enzymes, such as the lipase from *Pseudomonas cepacia* from Amano [21, 22].

In 2007, we published a proof of concept, showing that the DFS produced by cultivating *B. contaminans* LTEB11 on a mixture of corn bran and corn oil (5% v/m) could be used to catalyze the synthesis of biodiesel esters by esterification or transesterification [1]. The reactions were done in a reaction medium containing *n*-heptane as a cosolvent, with the intention of minimizing denaturation of the lipase. This work made two key contributions. First, we described the use of a bacterium that produces relatively high amounts of lipase in SSF (108 U of *p*NPP-hydrolyzing activity per gram of dry solids after 72 h). At that time, most papers dealing with the production of lipases by SSF had involved filamentous fungi. Second, we demonstrated that it was possible to use lyophilized fermented solids containing lipases to catalyze ester synthesis reactions.

Soon afterward, the paper of Martínez-Ruiz et al. [6] appeared. These authors cultivated *Rhizopus* sp. on perlite impregnated with a nutrient solution containing olive oil, lactose, and urea, as major components, and polyvinyl alcohol and various mineral salts, as minor components. After the fermentation, the solids were either lyophilized or air dried, with both methods giving DFS with similar olive-oil-hydrolyzing activities. These DFS were used to catalyze the esterification of oleic acid with ethanol using *n*-hexane as a solvent. With an ethanol to oleic acid molar ratio of 5:1 (250 mmol L^{-1} ethanol and 50 mmol L^{-1} oleic acid), a conversion of 98% was obtained after 60 min at 45°C.

2.3 Production of Biodiesel by Transesterification Using Bacterial Fermented Solids

Fernandes et al. [1] used the DFS produced by *B. contaminans* LTEB11 to catalyze ester synthesis by transesterification of corn oil with ethanol, in a medium containing *n*-heptane as a cosolvent. The reactions were done with 15 mL of reaction medium initially containing 70 mmol L^{-1} of corn oil. The best conversion after 120 h of

reaction at 37°C was 94.7%, obtained with an ethanol to oil molar ratio of 4.5:1 and the addition of DFS containing 43.9 U of *p*NPP-hydrolyzing activity. Since the corn oil concentration in *n*-heptane was relatively low, it limited productivity of the system. Salum et al. [15] therefore investigated the possibility of carrying out the transesterification in a solvent-free medium.

The transesterification reactor of Salum et al. [15] was a column packed with 3 g of DFS that was produced by cultivating *B. contaminans* LTEB11 on a mixture of sugarcane bagasse and sunflower seed meal. This substrate mixture gave a *p*NPP-hydrolyzing activity of 234 U g^{-1} after 96 h of cultivation, this being more than twice the activity of the DFS used by Fernandes et al. [1]. The column reactor was operated in batch mode, with closed-loop recirculation of a solvent-free reaction medium initially consisting of ethanol and soybean oil. A conversion of 95% after 46 h of reaction was obtained, at 50°C, using a molar ratio of ethanol to oil of 3:1 (the ethanol was added in two steps, at 0 and 7 h), with the addition of 1% (m/m) of water to the reaction medium. This stepwise addition of ethanol helps to avoid high ethanol concentrations in solvent-free media, thereby minimizing inhibition and denaturation [23, 24].

The conversion of 95% in 46 h obtained by Salum et al. [15] corresponds to an ester productivity of 152 mg g^{-1} h^{-1} (mass of esters produced per mass of DFS used per hour). This was a significant improvement over the process of Fernandes et al. [1], with the same conversion being achieved in less than half the time and with a more concentrated medium (i.e., a solvent-free medium). When Salum et al. [15] reused the DFS in successive 47-h transesterification cycles, conversions of over 95% were maintained for three cycles, but in the seventh cycle, the conversion was less than 60%.

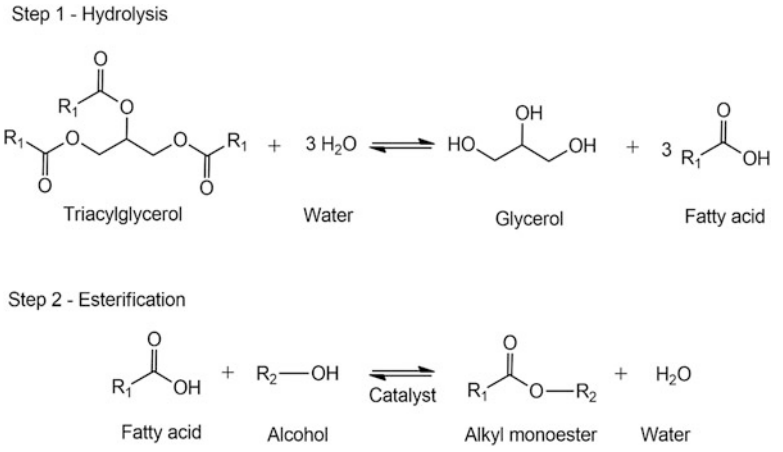
Other groups have used DFS as biocatalysts to produce biodiesel by transesterification, but they have not obtained results as good as those of Salum et al. [15]. Liu et al. [16] produced DFS by cultivating *Burkholderia cenocepacia* on a mixture of sugarcane bagasse and sunflower cake (5:3 m/m). Their DFS had an olive-oil-hydrolyzing activity of 72 U g^{-1}. They used their DFS to catalyze the transesterification of soybean oil with ethanol in a reaction medium containing *tert*-butanol (at a level of 40% (v/v) in relation to the oil content) (Table 1). Their best conversion was 86% after 96 h. They reused the DFS in three 96-h reaction cycles (giving a total of 288 h), obtaining a conversion of 67% in the last reaction cycle. They later optimized this reaction using a Box-Behnken factorial design. The best result was a conversion of 91% after 96 h, achieved at 44°C using a molar ratio of ethanol to oil of 3:1, a DFS concentration of 1.63 g mL^{-1}, with the addition of 4.6% (m/m) water and 20% (v/v) *tert*-butanol [17]. This result corresponds to an ester productivity of 87 mg g^{-1} h^{-1}.

Recently, our group investigated the transesterification of palm oil with ethanol in a solvent-free medium [20]. The production of the fermented solid by *B. contaminans* LTEB11 was optimized, giving 160 U g^{-1} of triolein-hydrolyzing activity. In shake flasks, a conversion of 90% in 48 h was obtained, using a molar ratio of ethanol to palm oil of 4.5:1 and 8% (m/m) of DFS in relation to the palm oil, with the ethanol being added in three steps in equal aliquots. In a packed-bed

bioreactor containing 12 g of DFS (12%, m/m, in relation to the palm oil mass), with recirculation of the reaction medium, we obtained a conversion of 89% in 30 h, this being achieved using a molar ratio of ethanol to palm oil of 5.5:1, with the addition of ethanol in four steps. The DFS was reused in successive 30-h cycles, with washing of the DFS between the cycles with *n*-hexane and *tert*-butanol. A conversion of 66% was obtained in the fifth cycle.

2.4 *Production of Biodiesel Esters by Esterification Using Bacterial Fermented Solids*

Most studies of the production of biodiesel by the enzymatic route have involved transesterification of oils. However, recently, there has been interest in lipase-catalyzed esterification of fatty acids, within the context of the so-called hydroesterification processes [7, 8, 25–28] (Scheme 2). The hydroesterification route for biodiesel production includes two steps. In the first step, triacylglycerols are hydrolyzed, either enzymatically or chemically, with subsequent recovery of fatty acids by distillation. In the second step, the fatty acids are esterified with a short-chain alcohol; likewise, this step can be catalyzed either chemically or enzymatically. Hydroesterification has several potential advantages over the traditional transesterification route for biodiesel production. First, lower-quality raw materials can be used, containing significant amounts of free fatty acids or water. These raw materials are significantly cheaper than the high purity vegetable oils typically used in transesterification processes [26, 27]. Second, glycerol is removed after the first step of the hydroesterification process, thereby avoiding the sorption of glycerol onto



Scheme 2 Production of biodiesel esters in a hydroesterification process. In the first step, a triacylglycerol is hydrolyzed to produce glycerol and fatty acids. In the second step, the fatty acids are esterified with an alcohol

solid catalysts, which can lead to decreased reaction rates due to mass transfer limitations [29, 30].

In our initial esterification studies with fermented solids, Fernandes et al. [1] used the DFS produced by *B. contaminans* LTEB11 to catalyze the esterification of oleic acid with ethanol in 5 mL of reaction medium containing 70 mmol L^{-1} of oleic acid dissolved in *n*-heptane as a cosolvent. After optimization, a conversion of 94% was obtained in 18 h at 37°C, using a molar ratio of ethanol to acid of 5:1 and with the addition of DFS containing 60 U of *p*NPP-hydrolyzing activity (corresponding to 0.55 g of DFS, which represented 2.8% m/m in relation to the oleic acid). Since the oleic acid concentration was relatively low, although high conversions were obtained, the productivity of this system was relatively low, only 22 mg g^{-1} h^{-1}. Subsequent esterification studies were done using solvent-free media.

Later, Soares et al. [7] used DFS of *B. contaminans* LTEB11, produced as described by Salum et al. [15], to catalyze the esterification of fatty acids with ethanol in a solvent-free medium. The fatty acids had been obtained through the hydrolysis of soybean soapstock acid oil in subcritical water. The reaction medium was circulated, in a closed-loop batch system, from a reservoir through a packed-bed bioreactor containing 12 g of DFS. The best conversion was 92% in 31 h, obtained at 50°C.

The work of Soares et al. [7] brought several advantages over the results for transesterification of soybean oil reported by Salum et al. [15]. First, a lower-grade starting material was used, namely, soybean soapstock acid oil. Second, the esterification process was 15 h shorter than the 46 h required for the transesterification process. Third, the DFS were obtained through a simple air drying, whereas Salum et al. [15] had lyophilized the fermented solids.

Soares et al. [7] reused the DFS in 48-h esterification cycles, maintaining conversions of over 80% during seven cycles. This performance is better than that which Salum et al. [15] obtained for repeated 47-h transesterification cycles with the same DFS, with less than 60% conversion in the seventh cycle.

Two important phenomena occurred in the studies of Soares et al. [7]: First, although the reaction medium was originally monophasic, a second liquid phase formed during the reaction, presumably due to the production of water by the esterification reaction and, second, part of the reaction medium remained sorbed by the fermented solid when the bioreactor was drained at the end of the experiment. These phenomena were then studied in greater depth by Soares et al. [31], who determined the compositions of the aqueous and organic phases of the bulk reaction medium and also of the phase sorbed onto the DFS, this being done for three experiments with different initial overall molar ratios of ethanol to fatty acid, 1:1, 1.5:1, and 3:1, designated as MR1:1, MR1.5:1, and MR3:1, respectively.

In the bulk reaction medium, the time required for the aqueous phase to appear increased as the initial molar ratio of ethanol to fatty acid increased. This is not surprising, as a higher concentration of ethanol in the initial organic reaction medium increases the miscibility of water in this medium. Once it formed, this aqueous phase contained almost no fatty acids (for which the mole fraction remained below 0.035%) and esters (for which the mole fraction remained below 1.2%). Although

this is the first time that the formation of an aqueous phase was observed for a system involving DFS, this phenomenon has been previously observed in other solvent-free systems in which immobilized lipases have been used to catalyze esterification of fatty acids [32–35].

An even more interesting result was that the phase sorbed onto the DFS represented up to 30% of the overall reaction medium and was predominantly polar: the water contents of the sorbed phase for MR1:1 and MR1.5:1 remained above 50%, while the ethanol content of the sorbed phase for MR3:1 remained above 65% [31]. On the other hand, at all times in all three experiments, the sorbed phase had a fatty acid content of less than 8 mol% of fatty acids. As a result of these sorption phenomena, the sorbed phase had molar ratios of ethanol to fatty acid that were significantly higher than those found in the bulk reaction medium: For MR1:1, the molar ratio in the sorbed phase remained above 2:1; for MR1.5:1, the molar ratio in the sorbed phase remained above 4:1; finally, for MR3:1, the molar ratio in the sorbed phase remained above 27:1. The formation of a sorbed phase on immobilization supports had been observed previously. For example, nonpolar organic components sorb onto the poly-(methyl methacrylate) beads used in Novozym 435 [36], while water sorbs onto the macroporous anionic resin (Duolite A568) used in Lipozyme [32]. However, the sorption of medium components on DFS had not previously been studied.

The high ethanol contents found by Soares et al. [31] for the phase sorbed on the DFS may explain the results obtained by Dias [37], who used a single-pass continuous system, comprising three packed-bed bioreactors in series, each containing 40 g of DFS, produced using *B. contaminans* LTEB11. The solvent-free reaction medium fed to the system had a molar ratio of ethanol to fatty acid of 3:1, in an attempt to maximize conversion. However, although the first reaction medium to pass completely through the system had a conversion of 46% (after a total residence time of about 4 h), 14 h later, the conversion of the medium exiting the system had fallen to around 10%. The most likely explanation is that, as the medium passed through the columns, a significant amount of ethanol sorbed onto the DFS, favoring denaturation of the lipase.

Given the poor performance of the single-pass continuous system, Dias et al. [10] scaled up the process for the production of biodiesel by using a closed-loop batch system with recirculation, as Soares et al. [7] had done. The packed-bed bioreactor contained 120 g of DFS produced by *B. contaminans* LTEB11, while the initial reaction medium contained 245 g of ethanol and 1,000 g of olein, which contains 60% oleic acid and 22% linoleic acid. This reaction medium had an initial molar ratio of ethanol to fatty acid of 1.5:1. A conversion of 88% in 24 h was achieved, with the use of a decanting reservoir with two compartments. In this reservoir, the reaction medium returns to the first compartment and then overflows into a second compartment from which the medium is recirculated back to the packed-bed bioreactor. When the system becomes biphasic, the denser aqueous phase is retained at the bottom of the first compartment, thereby decreasing the water content of the medium fed back to the bioreactor. This system was used in six successive 48-h batches with the same DFS, in which a total of 4.6 kg of ester was produced [10]. To

date, this is the largest scale at which biodiesel has been produced using DFS, via either esterification or transesterification.

Serres et al. [38] developed a mathematical model to describe the reaction profiles obtained by Soares et al. [31]. This model recognized that the reaction involves both adsorption and reaction steps. Adsorption was described by Langmuir isotherms in multicomponent systems. In this case, the number of moles of component i sorbed onto the DFS (denoted S_i) is given by

$$S_i = \frac{a_i B_i}{1 + \sum_{j=1}^{n} \left(b_j B_j\right)}$$

where B_i is the number of moles of component i in the bulk phase, a and b are sorption parameters, and n is the number of medium components, including both substrates and products. A sensitivity analysis showed that the sorption of the products was not important in the denominator, such that the equation describing sorption simplified to:

$$S_i = \frac{a_i B_i}{1 + b_{\mathrm{FA}} B_{\mathrm{FA}} + b_{\mathrm{Et}} B_{\mathrm{Et}}}$$

There were four such equations, one each for the fatty acid (FA), ethanol (Et), water (W), and Ester (Es).

The rate of reaction (r) was expressed in terms of the concentrations of the components in the sorbed phase. After the sensitivity analysis, the "Ping Pong bi bi" equation was simplified to

$$r = \frac{(k_{\mathrm{cat}} S_{\mathrm{FA}} S_{\mathrm{Et}} - \alpha S_{\mathrm{W}} S_{\mathrm{Es}}) E}{D_1 S_{\mathrm{Et}} + S_{\mathrm{FA}} S_{\mathrm{Et}} + D_2 S_{\mathrm{Es}}}$$

In this equation, k_{cat} is the turnover number of the enzyme, and the constants α, D_1, and D_2 are fitting parameters that represent combinations of fundamental kinetic constants. E is the fraction of active enzyme (relative to the initial amount of active enzyme). It denatures according to first-order decay kinetics:

$$\frac{dE}{dt} = -k_{\mathrm{d}} E$$

The first-order denaturation constant, k_{d}, was assumed to depend on the sorbed ethanol according to a sigmoidal relationship:

$$k_{\mathrm{d}} = \frac{k_{\mathrm{dmax}} (S_{\mathrm{Et}})^m}{K_{\mathrm{D}} + (S_{\mathrm{Et}})^m}$$

In this equation, k_{dmax}, K_{D}, and m are empirical fitting constants.

With a single set of parameters, this model was able to fit well to the experimental data profiles of Soares et al. [31]. It not only described the profile for overall percentage conversion, but it also described the profiles for the compositions of the bulk phase and sorbed phases. This opens up the possibility of using the model as a tool to guide the scale-up and optimization of operation of the closed-loop bioreactor system. However, in order to describe the separation of phases in the two-compartment decanting reservoir, it is necessary to characterize the phase behavior of the system components. The necessary phase diagrams were obtained for the system FA-SSAO (fatty acids from soybean soapstock acid oil), ethanol, water, and esters by Serres et al. [39]. However a complete model of the system has not yet been developed.

2.5 *Production of Biodiesel Using* Rhizopus microsporus *Fermented Solids*

Dry fermented solids produced using fungal strains have also been used to produce biodiesel. Our investigations into the use of fungal strains were motivated by the fact that *B. contaminans* belongs to the *Burkholderia cepacia* complex, which is a group of species of *Burkholderia* that are opportunistic pathogens of humans. Although the fermented solids produced by *B. contaminans* showed good potential in biodiesel synthesis, as described in the previous section, the production and application of fermented solids containing *B. contaminans* would require special containment procedures and equipment in order to protect the process workers. This would significantly increase process costs. Our reasoning was that the need for special containment could be avoided by using a nonpathogenic lipase-producing fungus, thereby reducing costs.

In our initial studies, we grew a strain of *Rhizopus microsporus* (CPQBA 312-07 DRM) on the same 1:1 mixture (on a dry mass basis) of sugarcane bagasse and sunflower seed meal that Salum et al. [15] had used to produce fermented solids with *B. contaminans* [18]. The tricaprylin-hydrolyzing activity of these DFS was 91 U g^{-1}. However, a much higher tricaprylin-hydrolyzing activity of 183 U g^{-1} was obtained with an improved medium comprised of sugarcane bagasse impregnated with an emulsion. This emulsion was prepared using soybean oil and a nutrient solution containing urea, lactose, and various mineral salts. The improved DFS gave a conversion of 68% in 72 h for the transesterification of corn oil with ethanol in a solvent-free medium, with stepwise addition of the ethanol (three equal aliquots at 0, 24, and 48 h). This performance is significantly lower than the 95% conversion after 46 h that we obtained previously for the transesterification of soybean oil in solvent-free medium using the DFS of *B. contaminans* [15]. The lipase activity in the *R. microsporus* DFS was highly sensitive to denaturation by the ethanol in the transesterification reaction medium, with final conversions below 10% being obtained when the ethanol was added in a single step at the beginning of the reaction.

The same improved *R. microsporus* DFS gave better conversions for biodiesel synthesis via esterification [13]. With an overall molar ratio of ethanol to fatty acid of 10:1 (equal aliquots added at 0 and 24 h), the conversions at 48 h were 98% for oleic acid and 86% for fatty acids obtained through the hydrolysis of soybean soapstock acid oil in subcritical water.

The good results that we obtained for the esterification motivated us to scale up the production of the DFS [9]. We used a pilot-scale SSF bioreactor [40] containing 15 kg of a 1:1 mixture (on a dry mass basis) of wheat bran and sugarcane bagasse, with the addition of urea as an additional nitrogen source. The peak olive-oil-hydrolyzing activity of the DFS produced in the pilot bioreactor was 113 U g^{-1} (at 20 h), which is much lower than the value of 265 U g^{-1} obtained (at 18 h) in a laboratory-scale packed-bed bioreactor containing only 10 g (dry mass) of the same substrate. Despite this difference in hydrolytic activities, both fermented solids gave a conversion of 69% in 48 h for the esterification of oleic acid with ethanol in a solvent-free system [9]. This conversion is lower than the value of 98% in 48 h obtained by Botton et al. [13] for the improved DFS produced at laboratory-scale (see the previous paragraph); however, we have not yet used the improved solid medium in the pilot bioreactor.

2.6 *Production of Biodiesel Using* Rhizomucor miehei *Fermented Solids*

A group at the Federal University of Rio de Janeiro has investigated the production of biodiesel esters using DFS produced by cultivating the fungus *Rhizomucor miehei* on babassu cake. These DFS will hereafter be referred to as $DFS_{RM\text{-}BC}$. Initially, they developed a hydroesterification process in which acid oil from macaúba (*Acrocomia aculeata*) pulp was hydrolyzed using a lipase from dormant seeds of the castor bean (*Ricinus communis*), with the liberated fatty acids being esterified with ethanol in a solvent-free system using $DFS_{RM\text{-}BC}$ [8]. In the esterification step, a conversion of 91% was obtained in 8 h, using stepwise addition of ethanol (at 0, 1 and 2 h). $DFS_{RM\text{-}BC}$ gave conversions of over 60% when it was reused in eight successive 6-h esterification reactions.

$DFS_{RM\text{-}BC}$ was also used to catalyze the esterification of palm fatty acid distillate and soybean fatty acid distillate, these raw materials being by-products of the refining of palm oil and soybean oil, respectively. For both raw materials, conversions around 70% were obtained in 6 h in solvent-free medium, with either ethanol or methanol as the alcohol [11]. In the studies of the reutilization of $DFS_{RM\text{-}BC}$ in successive 6-h cycles, Aguieiras et al. [11] washed the $DFS_{RM\text{-}BC}$ between cycles with a solvent. The idea was to remove the residual reaction medium from the $DFS_{RM\text{-}BC}$, since sorbed free fatty acids might cause mass transfer limitations, decreasing the performance of the biocatalyst. For both raw materials, when $DFS_{RM\text{-}BC}$ was washed with *n*-hexane, conversions around 70% were maintained

over five cycles. On the other hand, when $DFS_{RM\text{-}BC}$ was washed with ethanol between cycles, conversions around 70% were maintained over five cycles only for the esterification of soybean fatty acid distillate; for palm fatty acid distillate, the conversion in the fifth cycle was around 35%. Aguieiras et al. [11] explained these results by pointing out that ethanol can remove unsaturated free fatty acids, which represent 75% of the fatty acids in the soybean fatty acid distillate, but not palmitic acid, which represents 47% of the fatty acids in the palm fatty acid distillate.

$DFS_{RM\text{-}BC}$ have also been used to catalyze the transesterification of fatty acid esters from macaúba acid oil with several alcohols (methanol, ethanol, isopropanol, and *n*-butanol) in a solvent-free system, with ultrasound irradiation [19]. The best result was obtained for butyl ester, with 79% conversion after 72 h. The DFS kept 55% of their initial activity after four cycles of reuse.

Finally, $DFS_{RM\text{-}BC}$ have been used to catalyze the simultaneous esterification and transesterification of macaúba acid oil with anhydrous ethanol in a solvent-free system [12]. After 96 h, the conversion was 79%, with the free fatty acids having decreased from an initial value of 29% to 3%. The used $DFS_{RM\text{-}BC}$ were removed, and a new batch of $DFS_{RM\text{-}BC}$ was added to the reaction medium. After a further 96 h reaction cycle, the conversion had increased to 91%. Better conversions might be possible if this system were optimized, or the product could be purified to remove residual mono- and diglycerides. Simultaneous transesterification and esterification of macaúba acid oil in a solvent-free system was also achieved with a fermented solid produced by cultivating *R. miehei* on cottonseed meal [14]. Conversions of about 76% were achieved in 24 h with either methanol or ethanol as the alcohol.

3 Miscellaneous Applications of Dry Fermented Solids

Although most studies of the application of DFS have focused on the synthesis of biodiesel, there have been some studies into other applications aimed at producing organic compounds (Table 2) [2, 41–44]. These other applications include synthesis reactions, such as the kinetic resolution of racemic alcohols [2, 42], the synthesis of modified lipids [41], and the synthesis of sugar esters [44], and hydrolytic reactions, such as the hydrolysis of pectin to produce D-galacturonic acid [43]. DFS have also been used to hydrolyze lipids in high-fat wastewaters [45–50]; however, this application is beyond the scope of this review.

3.1 Use of Fermented Solids in the Resolution of Racemic Mixtures

Nagy et al. [2] screened DFS produced with 38 strains of filamentous fungi for lipase activity and enantioselectivity against secondary racemic alcohols. The fermented solids were produced using a mixture of wheat bran and olive oil (9:1 m/m), wetted

Table 2 Studies of the direct application of dry fermented solids to obtain different products

Substrate used to produce the fermented solid	Microorganism	Application	Substrates of the reaction	Solvent	Bioreactor	Conversion in time taken	Reference
Wheat bran	Various filamentous fungi	Kinetic resolutions of racemic secondary alcohols	*rac*-enylethanol or *rac*-1-cyclohexylethanol or *rac*-1-(naphth-2-yl)ethanol and vinyl aa *rac*-1-phenylethanol or *rac*-1-cyclohexylethanol or *rac*-1-(naphth-2-yl)ethanol and vinyl acetate	*n*-hexane	Shake flasks	Conversions as high as 50% obtained, with enantioselectivity for the R-isomer (E_R) over 100	[2]
Sugarcane bagasse and sunflower seed meal	*Rhizopus oryzae*	Interesterification	Palm stearin, palm kernel oil and a concentrate of triacylglycerols enriched with ω-3 polyunsaturated fatty acids	Solvent-free	Shake flasks	Solid fat content (at 35°C) as low as 2.3% obtained in 24 h	[41]
Sugarcane bagasse and nutrient solution	*Rhizopus microsporus*	Kinetic resolution of *rac*-1-phenylethanol	*rac*-1-phenylethanol and isopropenyl acetate	*n*-heptane	Shake flasks	23% in 96 h with enantioselectivity for the S-isomer (E_S) of 26	[42]
Sugarcane bagasse and orange peels	*Aspergillus oryzae*	Hydrolysis of pectin to liberate D-galacturonic acid	Concentrated pectin solution (10% m/v)	Solvent-free	Shake flasks	60% of the D-galacturonic acid present in the pectin liberated in 48 h	[43]
Sugarcane bagasse, nutrient solution, and soybean oil	*Burkholderia contaminans*	Regioselective modification of carbohydrates	Methyl-α-D-glucopyranoside and vinyl acetate	Solvent-free	Shake flasks	76% in 72 h	[44]

with a salt solution to a moisture content of 60% or 70% (m/m). All strains produced DFS that were enantioselective, most for the *R* isomer, following the Kazlauskas rule for secondary alcohols [51]. For these strains, conversions of the secondary alcohol (*R,S*)-1-phenyl-1-ethanol were near 50%, with enantiomeric ratios ($E_R = (k_{catR}/K_{MR})/(k_{catS}/K_{MS})$) above 200. The exception was the DFS produced with *Mucor hiemalis*, which was enantioselective for the *S* isomer. In this case, the conversion of (*R,S*)-1-phenyl-1-ethanol was 14%, with an E_S value ($((k_{catS}/K_{MS})/(k_{catR}/K_{MR}))$ of 15.

Recently, our group also produced a DFS that has preference for the *S* isomer of secondary alcohols [42]. This DFS was produced by cultivating *R. microsporus* on sugarcane bagasse impregnated with olive oil and urea, with an initial moisture content of 80% (wet basis). Through optimization of the resolution reaction, we increased the conversion of (*R,S*)-1-phenyl-1-ethanol from 3% to 23%, achieving an E_S value of 26. This result is better that the results obtained with (*R,S*)-1-phenyl-1-ethanol by Nagy et al. [2] using the DFS of *M. hiemalis* (conversion of 14%, $E_S = 15$).

3.2 Use of Fermented Solids in Interesterification Reaction to Produce Modified Fats

Taking advantage of the fact that the lipases produced by the genus *Rhizopus* are *sn*-1,3 specific and that species of this genus are considered GRAS, Rasera et al. [41] used DFS produced by *R. oryzae* to catalyze the interesterification of palm stearin, palm kernel oil, and a concentrate of triacyclglycerols enriched with omega-3 polyunsaturated fatty acids (ω-3 PUFAs) (EPAX 4510TG), in a solvent-free medium. The aim was to incorporate ω-3 PUFAs into the palm stearin and palm kernel oil, thereby reducing the solid fat content at 35°C (SFC35°C) of the blend. For table margarines, this value must be as low as possible, to ensure that the margarine melts in the mouth, avoiding a coarse and sandy texture [52]. The initial blend had an SFC35°C value of 12.3%. The best conditions for interesterification were 65°C, a palm stearin content of 38% and an EPAX content of 15%. Under these conditions, after 24 h, the product had an SFC35°C of 2.3%, making it suitable for the production of margarines and shortenings [41]. Despite this success, a key challenge is to reduce the reaction time, since similar reductions can be obtained in as little as 30 min using commercial lipases such as Lipozyme TL IM [53].

3.3 Use of Fermented Solids for the Production of High-Value Sugar Esters

Recently, Villalobos et al. [44] showed that the DFS produced by *B. contaminans* LTEB11 can be used to produce high-value sugar esters. The DFS were produced on sugarcane bagasse impregnated with a solution containing urea, lactose, and a micronutrient solution and 20% of soybean oil (w/w, on a dry basis). They had an

olive-oil-hydrolyzing activity of 160 U g^{-1}, which is 1.5-fold higher than the activity of the DFS produced by Soares et al. [7] with the same bacterium cultivated on a mixture of sugarcane bagasse and sunflower seed meal. The DFS were used to catalyze the transesterification of methyl-α-D-glucopyranoside (α-MetGlc) with vinyl acetate in a solvent-free medium. In initial studies done with 4 mL of a reaction medium containing 200 mg of DFS, 4.5 mg of α-MetGlc, and vinyl acetate, the main product obtained was methyl 6-O-acetyl-α-D-glucopyranoside (6-OAc-α-MetGlc), with a conversion of α-MetGlc to 6-OAc-α-MetGlc of 65% at 72 h. This system was scaled up to 1 L of reaction medium. A conversion of 76% was obtained at 72 h, with a calculated yield of 1.0 g (4.43 mmol) of 6-OAc-α-MetGlc. This is the largest scale reported to date for production of sugar esters by enzymatic catalysis, in terms of moles of sugar ester formed, indicating that the use of DFS has good potential in this system.

3.4 Use of Fermented Solids in the Production of D-Galacturonic Acid

Leh et al. [43] produced DFS containing pectinase activity by cultivating *Aspergillus oryzae* on a mixture of ground sugarcane bagasse and orange peels (30:70 m/m). The DFS were added to a 10% m v^{-1} pectin solution, giving a concentration of 247 mmol L^{-1} of D-galacturonic acid at 35 h. This is the highest D-galacturonic acid concentration reported for enzymatic pectin hydrolysis to date and represents a conversion of 60%, based on the D-galacturonic acid content of the original pectin. This opens the possibility of a cheap route for producing D-galacturonic acid from pectin in a citrus waste biorefinery. The D-galacturonic acid can then serve as the basis for the production of several useful organic chemicals [54].

4 General Considerations

As our review has shown, DFS containing naturally immobilized enzymes (lipases and pectinases) have been produced using both bacteria and fungi and then used in synthesis and hydrolysis reactions to produce organic compounds of biotechnological interest. To date, the main application of DFS has been in the production of biodiesel, by transesterification and esterification, in solvent-free media. Although many of the results reported in this review are promising, there are several hurdles to overcome before the processes can be scaled up successfully to industrial scale. We discuss the main hurdles below.

In general, processes involving DFS require longer reaction times than do processes that use commercial enzyme preparations. Although DFS are cheaper, the resulting low volumetric productivities will diminish the competitivity of processes using DFS as the biocatalyst. One key challenge is to decrease reaction times by increasing the enzymatic activity produced in the solids. Another key challenge is to improve the stability of the DFS so that they can be reutilized over many cycles.

To date, most processes have shown a significant drop-off in performance over ten or fewer reaction cycles.

The drying of the fermented solids should be done by air-drying, as lyophilization will increase the costs of producing DFS significantly. Air-drying protocols need to be optimized to minimize activity losses during this step. To reduce process costs, one possibility is to dry the solids directly in the bioreactor used for the synthesis or hydrolysis process. The stability of the DFS during storage also needs to be studied.

In hydrolytic reactions performed in aqueous media, the enzymes can leach from the DFS into the reaction medium. This is acceptable if simultaneous extraction and hydrolysis is desired. However, if one desires to maintain the enzyme in the DFS, an in situ immobilization procedure will be necessary. To date, there are no reports of such a procedure having been used with DFS.

To date, processes involving DFS have been applied at relatively small scales, involving, at most, little more than 100 g of DFS. Processes need to be developed not only to produce several kilograms of DFS but also to apply kilogram quantities in the biocatalytic process. However, one should carry out an economic feasibility study before one decides to scale up a process based on DFS. Only with such studies will it be possible to confirm whether the low costs of producing DFS offset their relatively low activities when compared to commercial enzymes. Such studies should also take into account the need to dispose of the DFS adequately after they have been used.

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Solid-State Anaerobic Digestion for Waste Management and Biogas Production

Haoqin Zhou and Zhiyou Wen

Contents

Abstract Solid-state anaerobic digestion (SS-AD) is commonly used to treat feedstocks with high solid content such as municipal solid waste and lignocellulosic biomass. Compared to liquid state anaerobic digestion (LS-AD), SS-AD has multiple advantages including high organic loading, minimal digestate generated, and low energy requirement for heating. However, the main disadvantages limiting the efficiency of SS-AD are long solid retention time, incomplete mixing, and an

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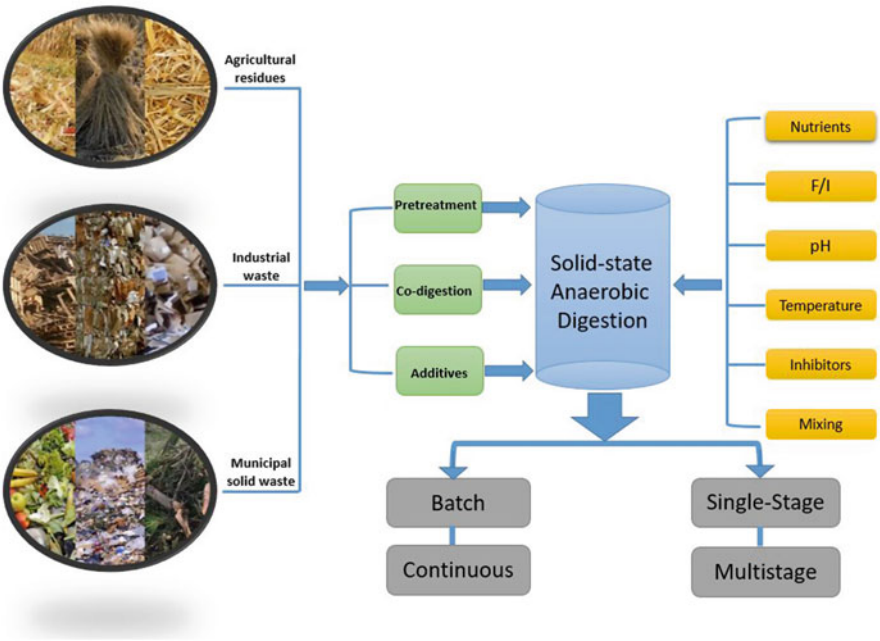
accumulation of inhibitors. For a successful and efficient SS-AD, it is important to control operation parameters such as nutrient levels, C/N ratio, feedstock-to-inoculum ratio, pH, temperature, and mixing. Biogas production in SS-AD performance can be enhanced by feedstock pretreatment, co-digestion, and supplement of additives such as biochar. The aim of this chapter is to provide a comprehensive summary of the current development in SS-AD as an effective way for treating solid waste materials.

Graphical Abstract



Keywords Biogas, Co-digestion, Pretreatment, Solid-state anaerobic digestion, Solid wastes

1 Introduction

With the growth of world population and economics, the production of solid wastes is increasing tremendously. A large quantity of these waste materials is biodegradable agricultural residues and municipal solid wastes (MSW). It is estimated that the annual production of MSW will reach 2.2 billion tons by 2025 [1]. These abundant

materials can be used as a feedstock for anaerobic digestion (AD) to produce energy while solving waste disposal problems.

AD is a process in which microorganisms decompose organic matters to produce biogas in the absence of oxygen. An AD process typically consists of four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In the hydrolysis stage, macromolecules such as cellulose, starch, proteins, and lipids are decomposed into monomers such as sugars, amino acids, and fatty acids. Those monomers are then converted into C2–C5-based volatile fatty acids (VFAs) and alcohols, as well as H_2 and CO_2 in the acidogenesis stage. In the acetogenesis stage, VFAs and alcohols are converted into acetate. In the methanogenesis stage, methane (CH_4) is produced through the conversion of acetate to CH_4 and CO_2 (acetoclastic methanogenesis) or the reduction of formate or CO_2 to CH_4 (hydrogenotrophic methanogenesis). Among these four steps, hydrolysis is commonly the rate-limiting step particularly when the feedstock is the complex organic substrates. When easily digestible organic matters are used as a feedstock, methanogenesis becomes the limiting step [2]. The biogas produced from an AD process usually contains 60–70% CH_4 and 20–30% CO_2 with trace amounts of ammonia, hydrogen sulfide, and hydrogen. The biogas can be combusted to generate heat and/or electricity or upgraded and refined into transportation fuels. Meanwhile, the digestate rich in nutrients, such as nitrogen and phosphorus, can be recycled as fertilizers or processed into biochar that can be used as soil amendment [3].

Based on the total solid (TS) content, AD can be defined as liquid state AD (LS-AD) with TS less than 15% or solid-state AD (SS-AD) with TS greater than 15% [4]. LS-AD is used to treat high moisture substrates such as animal manures and sewage. However, the large amount of water used in LS-AD process leads to a decreased volumetric CH_4 productivity and creates the problem of disposing large amount of digestate [5]. On the contrary, SS-AD can handle feedstocks with high organic loading with minimal water demand and results in a high volumetric CH_4 productivity. The wastewater generated and heating energy required in SS-AD are also reduced. However, due to inadequate mass transfer, SS-AD has disadvantages such as longer retention time, high cost, and a tendency to accumulate inhibitors [6]. In the past decade, a steady increase of publications in SS-AD indicates a great interest in this area (Fig. 1). The aim of this chapter is to provide a comprehensive review of the recent advances of SS-AD including feedstock, inoculum, factors affecting SS-AD performance, operation mode, and digestion process enhancement.

2 Feedstocks

Feedstocks with high moisture content, such as animal manure or municipal sewage, have been traditionally treated with LS-AD. Recent development of AD has expanded to feedstocks with high solid content such as agricultural residues (e.g., corn stover, wheat straw, and rice straw), industrial wastes, and municipal solid wastes; SS-AD has been increasingly used to treat these feedstocks. Corn stover,

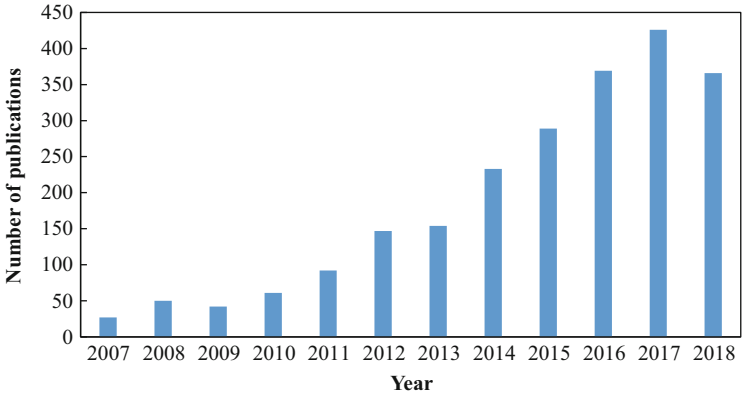


Fig. 1 Number of publications with keywords, "solid-state anaerobic digestion," via Google Scholar. The search included patents but did not include citations. Updated on September 16, 2018

with a 1:1 weight ratio of residue to grain [7], is the most abundant agricultural residue in the United States, with approximately 80 million dry tons of corn stover produced each year [8]. It has been reported that SS-AD (18% TS) treatment of corn stover produced a higher CH_4 yield than LS-AD (5% TS) [9, 10]. SS-AD of wheat straw, another abundant agriculture residue, also resulted in a much higher CH_4 yield than LS-AD [8, 11].

The microstructure of the fibrous feedstock significantly affects SS-AD performance. Cui et al. [10] examined the fiber structure in wheat straw by scanning it with an electron microscope (SEM). Compared to the raw wheat straw with long and smooth intact fibers, the spent wheat straw with rough fiber and serrations at the edge was more digestible. Similarly, corn stover treated by sodium hydroxide was more digestible than the raw corn stover [11].

The organic municipal solid wastes (OMSW), such as food and yard wastes, have also been commonly used as feedstock for SS-AD. It is estimated that 1.3 billion tons per year of food wastes are produced worldwide [12]. In the United States, food waste accounts for 12% of total municipal solid waste [13]. Food waste composition varies widely depending on geographical locations and the eating habits of local populations. In general, food wastes containing soluble organic matters can be easily converted into VFAs, which may inhibit the subsequent CH_4 formation if VFAs are overproduced. A two-phase SS-AD can successfully overcome this problem [14]. Among the 31 million dry tons per year of yard wastes generated in the United States, more than 60% were treated through composting, during which energy was wasted as respiration heat [5]. SS-AD as an alternative to composting can recover energy. However, the types of yard wastes affect the methane yield due to different TS, VS, and C/N in those materials [15, 16].

3 Inoculum

Inoculum brings the microbes, nutrients, and water to SS-AD reactors. Typical inoculum in SS-AD includes sewage sludge, ruminant cultures, and digested manure [17]. Since most solid feedstock does not naturally contain methanogens, methanogens-rich inoculum is essential to a SS-AD process [15]. Characterization of the microbial community in inoculum is important for an insightful understanding, particularly the functional partitioning of a SS-AD process. Shi et al. [16] studied the microbial community in SS-AD of corn stover using denaturing gradient gel electrophoresis and found enriched archaeal and bacterial communities in the system. In SS-AD of rice straw, a high-throughput sequencing analysis revealed that *Methanobacteria*, *Bacteroidia*, *Clostridia*, *Betaproteobacteria*, and *Gammaproteobacteria* were the primary species [18]. The acetoclastic *Methanosarcina* and hydrogenotrophic *Methanoculleus* coexisted in this system. For example, in the first 20 days of AD, *Methanosarcina* accounted around 86.5% of microbial population, while *Methanoculleus* accounted 32.1% of microbial population from days 7 to 45 [18]. Bacteria producing low temperature-adaptive lipases, *Psychrobacter*, was identified in SS-AD of a mixed kitchen waste, pig manure, and the sludge [19]. In SS-AD of fruit waste, a three-stage system was developed to accommodate the favorable conditions for hydrolysis, acidogenesis, and methanogenesis [20]. *Lactobacillaceae* and *Pseudomonadaceae* were predominant in the hydrolysis of carbohydrate into lactate and biomic acids, respectively. In the acidogenesis stage, the most abundant bacteria were switched to *Porphyromonadaceae* and *Enterobacteriaceae*, while methanogens were the dominant species in the methanogenic stage [20].

4 Factors Affecting Solid-State AD

4.1 Nutrients

Anaerobic microbes need balanced nutrients such as carbon, nitrogen, phosphorous, and minerals for their growth. Carbon, the primary energy source for cell growth, is usually rich in organic materials. Nitrogen and phosphorous are also essential for anaerobic microbes to synthesize proteins and nucleic acids, respectively. Ammonium is the nitrogen form methanogens can utilize [21] but will inhibit the microbial growth at high levels. The C/N ratio of the feedstock also plays an important role in digestion process. C/N ratio ranging from 20:1 to 30:1 with an optimal C/N ratio of 25:1 is recommended [22]. Too low C/N ratios increase the risk of ammonia inhibition, resulting in insufficient utilization of carbon sources, while an excessively high C/N ratio results in insufficient nitrogen to maintain microbial growth and biogas production. The demand of phosphorus is usually 15% of that of nitrogen [23].

Trace elements such as iron, cobalt, nickel, and sulfur are essential for methane fermentation [24–27]. Iron is often supplemented in AD systems to activate enzymes such as ATPase, PEP carboxylase, and serine transhydroxymethylase [28, 29]. Due to its reduction capacity, iron often reacts with sulfur to form FeS precipitant, reducing H_2S generation and alleviating odor problem [30]. Nickel is an essential element in coenzyme F430, hydrogenase, and CO dehydrogenase in methanogenic microbes [31–33]. Cobalt is involved in the activity of methyl transferase and CO dehydrogenase (CODH) in acidogenesis [31]. The addition of cobalt has been reported to stimulate CH_4 productivity in methanol LS-AD process [25]. Molybdenum is present in CO_2 reductase, a molybdoprotein that is responsible for reducing CO_2 to formate and subsequently reducing to CH_4 [34].

4.2 Feedstock-to-Inoculum Ratio

Feedstock-to-inoculum ratio (F/I) is another important factor in SS-AD. Too high F/I ratio could result in overproduction of VFAs due to excess organic loads, which eventually leads to an acidic pH and inhibition on methanogens. Zhou et al. [35] reported that the CH_4 yield of rice straw SS-AD was inversely proportional to F/I due to the VFAs accumulation and poor mass transfer. On the contrary, SS-AD of palm oil mill residues achieved the highest CH_4 production rates at the lowest F/I ratio within the range of 2:1–5:1, while a rapid hydrolysis at F/I ratio of 4:1–5:1 resulted in a VFAs accumulation and low CH_4 yield [36].

4.3 pH

The pH of a SS-AD system also affects the digestion performance. The ideal pH for a SS-AD process is within a narrow range of 6.8–7.2 [37]. However, different groups of microbes in SS-AD have different optimal pH requirements. For example, the optimal pH for acidogens is between 5.5 and 6.5, while methanogens are most active at pH 6.5–8.2 with an optimum at pH 7.0 [38]. Due to this discrepancy of pH requirement, two-stage SS-AD, i.e., separating acidogenesis and methanogenesis into two reactors, is usually used [37].

During an AD process, pH is affected by many parameters. In a SS-AD of OMSW with liquid digestate recirculation, the pH was low (<6.5) initially due to high VFAs concentration and then gradually increased to 8 after VFAs decreased from 12,000 to 1,000 mg/L within 1 week [39]. The buffer capacity of an AD system to resist pH fluctuation is evaluated through alkalinity. For example, in a corn stover SS-AD system with a less alkalinity (1,036 mg $CaCO_3$/kg), pH dropped from nine to below six rapidly with a decreased CH_4 yield [16]. When the alkalinity of the system was increased (>1,700 mg $CaCO_3$/kg) through adjusting the F/I ratio, pH of the same system was stabilized with only slight a decrease from 9 to 8.4 [16]. In order

to maintain a stable pH during SS-AD process, it is essential to balance VFAs concentration and bicarbonate. In general, reducing organic loading, adding bases or bicarbonates, and modifying F/I ratio are used to increase alkalinity in SS-AD systems [37].

4.4 Temperature

SS-AD is commonly operated at mesophilic (~37°C) or thermophilic (~55°C) conditions. Compared to the mesophilic AD, thermophilic AD has a shorter start-up time and hydraulic retention time (HRT) due to accelerated feedstock hydrolysis. The CH_4 yield in thermophilic SS-AD is also higher as methanogenic microbes have an optimal growth at 55°C [40]. Thermophilic AD can also produce pathogen-free digestate. Pohl et al. [41] compared the performance of wheat straw SS-AD under 37°C and 55°C. The CH_4 yield from the thermophilic AD was 36% higher than that in mesophilic AD due to a faster disintegration and hydrolysis of the feedstock.

However, compared to mesophilic SS-AD, poor stability and reliability often represent obstacles in thermophilic SS-AD. In general, microbes in thermophilic conditions are more sensitive to environmental changes, exhibiting poor stability and less diversity and richness in microbial community [38]. Also, the fast hydrolysis of feedstock in thermophilic processes often results in a rapid VFAs production, causing an imbalance between acidogenesis and methanogenesis. The higher temperature also shifts NH_3/NH_4^+ equilibrium toward the cytotoxic ammonia [40]. Heating energy in theomorphic AD is also higher [42]. Due to those reasons, theomorphic digesters are still not commonly used in commercial SS-AD.

4.5 Inhibitors

A variety of compounds have been reported inhibitory to SS-AD, causing an adverse shift in microbial population, an instability of the process, and a decreased CH_4 yield [43]. In LS-AD, the inhibitor concentrations can be diluted, while inhibitory effects in SS-AD cannot be alleviated and often cause severe inhibition to the system. The easily digestible feedstock often leads to a rapid hydrolysis and acidification, producing excessive VFAs which inhibit methanogens. For example, in SS-AD of tomato residues, VFAs concertation (12.48 g/L) was much higher than the threshold level (6 g/L) and caused CH_4 production inhibition [44]. Compounds derived from phenolic degradation, such as *p*-cresol, inhibit acetogenesis, resulting in accumulation of VFAs [45].

The partial pressures of CO_2 and H_2 in SS-AD system also affect the CH_4 production. Increasing CO_2 partial pressure results in an increased dissolved CO_2, which causes acidification and inhibition of methanogenesis. An elevated H_2 partial pressure leads to an accumulation of dissolved H_2, which inhibits the degradation of

VFAs [46]. In SS-AD of wheat straw, high H_2 partial pressure also led to a strong inhibition on the initial hydrolysis step [47]. Since both CO_2 and H_2 are needed to produce acetate/CH_4, a balanced CO_2/H_2 pressure in the headspace is essential to prevent inhibition.

Ammonia is produced from the degradation of nitrogenous compounds (e.g., protein and urea) during AD process. A moderate amount of ammonia is essential for bacterial growth and neutralizing VFAs to maintain a stable pH; however, excessive ammonia can inhibit methanogenesis. Ammonia exists as an equilibrium between ammonium ion (NH_4^+) and free ammonia (NH_3) [43]. Free ammonia can permeate cell membrane and cause proton imbalance and thus is inhibitory to microbial cells. Animal manure usually contains excessive ammonia, resulting in process inhibition. For example, in SS-AD of chicken manure, the digester was completely inhibited when influent total Kjeldahl nitrogen (TKN) (mainly ammonia) was 8.2 g/L [48]. After ammonia was removed from influent, the digester achieved a much higher CH_4 yield.

4.6 Mixing

A certain degree of mixing in SS-AD is necessary to enhance the transfer of organic substrates to microbes, prevent the sedimentation of denser particles or floating lighter materials, and facilitate the release of gas bubbles trapped in the solid feedstock. In SS-AD of rice straw, intermittent mixing with a 5/25 min on/off cycle at 160 rpm resulted in a good mass transfer while saving energy compared to a continuous mixing [35]. Premixing of the feedstock with inoculum is also needed before loading into SS-AD reactor [49, 50].

The methods of mixing in SS-AD can be liquid (leachate) recirculation, solid mixing using augers, and biogas recirculation [4], among which the leachate recirculation is commonly used. Leachate recirculation in SS-AD facilitates the nutrient diffusion from substrates to microbial cells [51] and also reduces the amount of inoculum as the microbe-containing leachate collected from the reactor can be reapplied to the digestion systems [52].

In addition to mixing, leachate recirculation also provides other benefits to SS-AD. For example, when leachate recirculation was used in the acidogenic reactor of a two-stage hybrid solid-liquid AD system, the extraction of organic matters from the feedstock was facilitated, and the pH was buffered [53]. In the SS-AD of hay and soybean processing wastes, leachate recirculation accelerated the daily CH_4 production to the peak value due to the enhancement of VFAs mass transfer from acidogenic to methanogenic pockets [54]. However, leachate recirculation may also lead to accumulation of VFAs and other inhibitors compounds; therefore, dilution of leachate with fresh water may be needed [15]. A leachate recirculation rate also needs to be carefully controlled to avoid irreversible acidification of the system [55].

5 Process Operations of SS-AD

5.1 Batch vs. Continuous Operations

Batch and continuous operations are two operation modes commonly used in SS-AD. Table 1 compares the performance of batch and continuous operation of SS-AD. Compared to continuous operation, batch operation is easier to maintain because it needs less capital and operating costs with less process control requirements. However, the biogas production in batch SS-AD is variable with time, and the majority of biogas is produced only at peak production time. For example, it was reported that in a 55-day batch SS-AD of corn stover, more than 80% of biogas was produced only at 36-day period of methanogenic phase [22]. Another limitation in batch SS-AD is the requirement of a large amount of inoculum (i.e., low F/I ratio). For example, Capson-Tojo et al. [56] reported that a batch SS-AD of food waste and cardboard mixture can only produce biogas at a F/I lower than 0.25; the biogas production had completely ceased when the F/I ratio was above this ratio due to overproduction of VFAs. Similar results were obtained for a batch operation of yard trimmings SS-AD process in which the highest CH_4 yield (244 L/kg VS) was obtained at the lowest level of the F/I ratio ranging from 0.2 to 2 [57]. The inoculum sources also significantly affected the batch SS-AD process. Guendouz et al. [58] studied three successive batches of MSW SS-AD and found that the second and third batches inoculated with the residue from the previous batch shortened the lag phase and accelerated reaction, which was due to the adaptation of the microbes to the digestion system.

Contrary to the batch operation, continuous SS-AD can consistently produce CH_4 at steady state. Organic loading rate (OLR), CH_4 production, and solid retention time (SRT) are the three main parameters in determining the interaction between microorganisms and substrates and thus are used in designing and evaluating a continuous SS-AD performance [51]. OLR represents the conversion capacity of an AD system; a maximum OLR level in SS-AD depends on various parameters such as reactor design, feedstock characteristics, microbial activity,

Table 1 Comparison of batch and continuous SS-AD systems

Parameters	Batch systems	Continuous systems
Investment	Low	High
Technical operation	Simple	Complex
Land acreage required	Large	Small
Organic loading rate (OLR)	Low	High
Inoculum	High	Low
Water consumption	Low	High
Biogas yield	Uneven; low	Even; high

temperature, pH, and toxicity level [59]. A high OLR is always preferred as it means an improved utilization efficiency and reduced digester size. However, high OLR can also lead to VFAs overproduction, causing an imbalance between acidogens and methanogens. For example, in a batch SS-AD process of rice straw, increasing TS loading from 20% to 24% prolonged the lag phase from 15 days to 20 days [35]. Similarly, increasing OLR from 2.3 to 9.2 kg VS/m^3 day in semicontinuous SS-AD of food waste slowed down bacteria acclimatization in the new environment, resulting in a prolonged adaptation time from 2 days to 31 days [60]. In a co-digestion of chicken manure and poplar leaf, CH_4 yield decreased when OLR increased from 4.0 to 8.0 g VS/L day [61].

One important operational parameter in continuous SS-AD is solid retention time (SRT); the time organic compounds stay in the digester. Due to slower mass transfer, the SRT in SS-AD is usually longer than the HRT commonly used in LS-AD [54]. The retention time needed for a complete degradation of solid feedstock can be determined through biomethane potential (BMP) assay [62]. An optimal SRT depends on many factors such as the feedstock, OLR, and TS. Decreasing SRT leads to washing out of microorganisms and insufficient substrate utilization. A longer SRT is usually not economical because it would require larger reactor volumes and higher costs for maintenance. SRT has a considerable impact on CH_4 production. In SS-AD of organic waste containing vegetable, fruit, and green waste, increasing SRT from 15 days to 35 days increased methane yield from 360 to 454 mL/kg VS [63].

5.2 Single-Stage vs. Multistage Operations

SS-AD can be operated in a single stage or multiple stages. In a single-stage system, the multiple steps of the conversion of organic substrates into biogas are implemented in one reactor vessel. In a multiple-stage operation, different conversion steps are implemented into different reactor vessels. A two-stage AD is commonly used as a multiple-stage operation during which the hydrolysis/acidogenesis is in the first reactor and the methanogenesis is in the second reactor [64].

Compared to a two-stage operation, a single-stage reactor is easier to design and build with less operating costs. However, the OLR in a single-stage digester is often limited in order to avoid VFAs overproduction and rapid pH drop [4]. Unlike the single-stage digester, two-stage systems can accommodate each conversion step, such as acidogenesis and methanogenesis, at their own optimal conditions (pH, temperature, OLR, and SRT). Two-stage systems generally perform better than a single-stage system. For instance, SS-AD of brewery spent grain (BSG) in a single-stage reactor was limited by the inhibitors, such as weak acids, furan derivatives, and phenolic substances, generated in the degradation of lignocellulose in BSG [65]. While in a two-stage SS-AD system, separating hydrolysis in one reactor and

Table 2 Biogas, methane production, and feedstock degradation of brewery spent grain SS-AD in single-stage and two-stage processes with raw and acid pretreated feedstock [65]

	Feedstock types	Single stage	Two stage
Biogas production (L/kg)	Raw	87.4	89.1
	Pretreated	89.1	103.2
Methane production (L/kg)	Raw	51.9	58.7
	Pretreated	55.3	58.7
Biodegradation %	Raw	62.0	63.5
	Pretreated	62.2	73.6

acidogenesis and methanogenesis in another granular-based reactor, both biogas production and feedstock biodegradation were improved (Table 2).

In some occasions, AD systems with more than two stages, such as three stages, are designed to create different favorable conditions for hydrolyzing bacteria, acidogenic bacteria, and methanogens, with each group of microbes performing a particular role. A three-stage system was used in the co-digestion of food waste and horse manure in which the first-stage hydrolysis and second-stage acidogenesis were operated as a solid state, while the methanogenesis was operated as a liquid state. This hybrid system increased CH_4 yield by 11.2–22.7% and the abundance of methanogenic archaea by 0.8–1.28 times compared to the single-stage reactor.

It should be noted that despite the fact that multistage AD systems are advantageous in improving AD performance, high capital and operating costs are the main hurdles for implementing this type of systems at a commercial scale. As a result, single-stage AD is still dominantly used. In Europe, for example, about 90% of the installed AD capacity is from single-stage systems, and only about 10% is from multistage systems [4].

6 Enhancement of Digestion Performance in SS-AD

6.1 Feedstock Pretreatment

As hydrolysis is the rate-limiting step for SS-AD of most solid feedstocks, various treatment technologies have been developed to accelerate the feedstock hydrolysis so overall biogas yield can be enhanced. Those pretreatment methods are summarized in Table 3.

6.1.1 Physical Treatment

Physical treatment such as milling and grinding reduces the particle size of the feedstock and thus provides a greater surface area for microorganisms to access. Tian et al. [66] reported a 29% increase in CH_4 yield in SS-AD of rape straw when the

Table 3 Methods used for the feedstock pretreatment in SS-AD

Treatment method	Processes	Feedstock	CH_4 yield (L/kg VS)	Enhancement (%)	References
Physical	Milling/grinding	Rice straw	188–243	29	[66]
	Sonolysis	OFMSW	186.4	16	[67]
	Microwave	Wheat straw	345	28	[68]
	Steam autoclaving	MSW	248	N/A	[69]
	Low temperature	High solid sludge	99.3–116 (biogas)	11%	[70]
Chemical	Alkaline	Poplar leaf	156.7	N/A	[61]
		Corn stover	372.4	37	[71]
	Peracetic acid oxidation	Waste activated sludge	175 (biogas)	21	[72]
	Ozonation	OFMSW	227.9	37	[67]
	Organosolv	Pinewood	71.4	84	[73]
Biological	*Trichoderma reesei*	Rice straw	214	78.3	[74]
	Pleurotus ostreatus	Rice straw	263	120	[74]
	Ceriporiopsis subvermispora	Yard trimmings	44.6	154	[75]
		Albizia chips	123.9	270	[76]
		Miscanthus sinensis	170–175	25	[77]
		Orange processing waste	275–330	N/A	[78]
	Stacking	Corn stover/ cow dung	450 (biogas)	29.1	[79]
	Pre-aeration	Rice straw	355.3	N/A	[35]
	Composting	Rice straw	353	N/A	[18]
Combined	Acid-thermal	Brewery spent grain	55.3	6.5	[65]
	Thermo-lime	*Spartina alterniflora*	218.1	N/A	[80]
	Milling-steam explosion	Birch wood	188.1	N/A	[81]

feedstock size was reduced from 2–2.5 cm to 0.5 mm. However, a too fine particle size may negatively affect the AD performance. For example, Motte et al. [82] compared the SS-AD of straw at three particle sizes (0.25, 1, and 10 mm) and found that the coarse particles (10 mm) resulted in the highest CH_4 yield followed with the medium size particles (1 mm) and the finest size (0.25 mm). The reason for this phenomenon was due to rapid acidification of the substrate at smaller particle sizes, which resulted in an overproduction of VFAs and rapid pH drop.

Thermal treatment is an effective treatment method for industrial SS-AD [83]. In addition to enhancing the reaction rate, thermal treatment removes pathogens, improves dewaterability, and decreases viscosity of the digestion system. In the SS-AD of steam autoclaved MSW, the digestate passed all the criteria for biosolids land application in the United States [69]. In the thermal treatment, an appropriate combination of temperature and time is needed as the high energy consumption often offsets the overall benefits. Liao et al. [70] studied the effect of treatment temperature (60, 70, and 80°C) on the SS-AD of sewage sludge and found that 70°C for 30 min was optimal for SS-AD. Under this condition, biogas yield increased by 11% and SRT reduced from 22 to 15 days.

Other physically based treatments were also reported. For example, ultrasound treatment generates both mechanical effects through cavitation and chemical effects through formation of free radicals. OMSW treated with low-frequency ultrasound released more soluble organic matters, resulting in a 16% increase in biogas production in SS-AD [67]. Microwave treatment is related to structure modification as well as thermal effects, contributing to increased sludge solubility [84], shortened initial lag phase [85], and improved CH_4 yield [68].

6.1.2 Chemical Treatment

Chemicals such as acids, alkaline, or oxidants can facilitate the breaking down of recalcitrant structures of feedstock. The effectiveness of chemical treatment relies on the feedstock characteristics and the reagents used. Feedstocks with easily digestible carbohydrates such as starch are typically not suited for chemical treatment because it accelerates starch degradation leading to VFAs overproduction and accumulation [86].

Alkaline treatment is usually carried out at ambient temperature with lime, sodium hydroxide, potassium hydroxide, and ammonium hydroxide as agents. The mechanism of alkaline treatment is to remove lignin from lignocellulose, improving the accessibility of hemicellulose and cellulose by the microbes and enzymes [87]. Additionally, the presence of residue alkali neutralizes carboxylic acids resulted from lignocellulose degradation in subsequent acidogenesis stage and prevents pH drop [71]. Zhu et al. [71] reported a 37% increase in biogas yield in SS-AD of corn stover treated with 5.0% NaOH compared to that of untreated corn stover. Liew et al. [88] achieved a 24-fold higher CH_4 yield in SS-AD of fallen leaves treated with 3.5% NaOH. However, excessive alkali loading may inhibit AD due to high pH or ion toxicity [89]. For instance, in SS-AD of corn stover, although the lignin degradation of corn stover increased with NaOH loadings from 1.0% to 7.5%, the biogas yield was not improved correspondingly; 7.5% NaOH loading actually inhibited biogas production due to VFAs accumulation and acidification [71].

Compared to alkali treatment, acid treatment is more effective to break down the recalcitrant lignocellulosic structure and produce reducing sugars [83]. However, compounds such as furfural and hydroxymethylfurfural (HMF) can be produced during acid treatment which inhibit the AD process [86]. Acid treatment also

requires additional bases to neutralize pH before starting SS-AD. Overall, acid treatment is less preferable than alkaline treatment in SS-AD.

Ozonation is another chemically based treatment in SS-AD with no chemical residues left in the system. As a strong oxidant, ozone decomposes into radicals and reacts with the soluble and insoluble fractions of the substrates [90]. The optimal ozone dosage is reported in the range of 0.05–0.5 g O_3/g TS [86]. In SS-AD of OMSW, a 37% increase in biogas yield was achieved with feedstock treated with ozone at 0.16 g O_3/g TS; however, higher ozone dosages (0.4 and 1.2 g O_3/g TS) led to a lower biogas yield, probably due to the formation of intermediate compounds that are difficult to be digested [67].

Organic solvent is another chemical used in the treatment of lignocellulose-based feedstock by removing lignin and thus improve degradability of lignocelluloses. For example, in SS-AD of elm, pine, and rice straw, treating the feedstock with ethanol prior to SS-AD enhanced CH_4 production by 73%, 84%, and 32%, respectively [73].

6.1.3 Biological Treatment

Biological treatment relies on microorganisms and/or enzymes to break down the recalcitrant structure of the feedstock. Enzymes such as peptidase, carbohydrase, and lipase [86] are commonly added to the LS-AD system to speed up the digestion. However, the practices of adding external enzymes to the SS-AD process have not been widely reported. Microorganisms, such as white-rot fungi, capable of decomposing lignin and altering the linkage between lignin and polysaccharides are commonly used in SS-AD [91]. The fungi *Pleurotus ostreatus* and *Trichoderma reesei* were used to decompose rice straw as an effective way to enhance CH_4 yield in SS-AD of this feedstock [74]. The white-rot fungus *Ceriporiopsis subvermispora* is considered one of the most effective species to degrade lignin while preserving cellulose [76]. Due to its selective degradation feature, *C. subvermispora*-treated SS-AD led to a 20.9% lignin degradation of yard trimming and only 7.4% cellulose degradation, achieving a 154% increase in CH_4 yield in the subsequent SS-AD [75]. When *C. subvermispora* was used to treat albizia chips, CH_4 yield increased 3.7-fold compared to the untreated feedstock [76].

Composting, an aerobic process facilitated by bacteria and fungi, is another biological treatment for SS-AD. Yan et al. [18] reported that composting rice straw resulted in a decrease of 63.6% TS, while the total carbon did not reduce significantly, proving that composting can effectively improve the biodegradability of rice straw. In order to improve the composting efficiency, pre-aeration is often used to generate enough self-heating to increase the temperature of OMSW for the start-up of thermophilic AD without external heating [92]. Composting with pre-aeration can also reduce the excessive organic compounds in feedstocks and thus reduce the risk of VFA overproduction and acidification in the following SS-AD [92]. However, excessive pre-aeration may cause toxic effect on methanogens by introducing oxygen. For example, Zhou et al. [35] reported that rice straw aerated for 2 days achieved the highest CH_4 yield, while the CH_4 yield gradually decreased when the aeration times increased from 4 days to 8 days.

6.2 Co-digestion

Co-digestion of different feedstocks is commonly used to adjust carbon-to-nitrogen (C/N) ratio of the substrates in SS-AD. Other advantages of co-digestion include improved nutrient profiles, a more balanced microbial community, obtaining a desired moisture content, and economic advantages by sharing equipment. However, there are several drawbacks of co-digestion such as the extra logistical cost of the different feedstock, premixing requirement, varied policy to control different waste materials, and increased effluent COD [93].

The optimal C/N ratio for an AD process is in the range of 20:1–30:1. Most lignocellulose has a higher C/N ratio than 30; therefore, blending lignocellulosic feedstock with animal manures (with a C/N ratio less than 20) is a good approach to balance C/N ratio of SS-AD system. Li et al. [61] reported co-digestion of poplar leaf (C/N = 35.4), and chicken manure (C/N = 8.09) brought C/N ratio to the optimal range (Table 4) and produced 15.28% more CH_4 than digestion of poplar leaf only.

In addition to adjusting C/N ratio, co-digestion of different feedstocks also provides other benefits such as better nutrients, diverse microorganism consortium,

Table 4 Co-digestion of different feedstock in SS-AD

Feedstock	Co-digestion feedstock	Mix ratio	C/N ratio	TS (%)	CH_4 yield (L/kg VS)	CH_4 increment (%)	Reference
Straw	Pig slurry	1:3 (weight)	41.3	20.7	240.8	N/A	[94]
Food waste	Horse manure	1:1 (weight)	20	20	370	N/A	[20]
Poplar leaf	Chicken manure	2:1 (VS)	21.9	22	115.7	15.28	[61]
Household organic waste	Cow manure	1:1 (weight)	11.1	15	247	10.7	[95]
Tomato residues/corn stover	Dairy manure	13:33:54 (weight)	25.1	20	415.4	50–1,020	[44]
Straw	Swine manure	1:0.23 (weight)	20	27	300	N/A	[96]
Food waste	Yard waste	1:9 (VS)	16.9	19.3	120	118	[97]
Food waste	Distiller's grains	1:8 (TS)	22.3	20	159.74	75.73	[98]
Spent mush-room substrate	Yard trimmings	1:1 (VS)	74.6	20	194	1,500	[99]
	Wheat straw	1:1 (VS)	71.9	20	269	2,200	
Expired dog food	Corn stover	1:1 (VS)	32.3	22	304.4	229	[100]
Biological sludge	OFMSW	1:4 (weight)	39.8	38.8	220.6	34	[101]

and stable pH and higher buffering capacity [61]. Khairuddin et al. [95] reported that the co-digestion of household organic waste and cow manure, even with a low C/N ratio of 11.1, still increased CH_4 yield by 10.7% compared to digestion of household organic waste only. Similarly, co-digestion of spent mushroom substrate and yard trimmings (with a C/N ratio of 74.6) produced 16-fold higher CH_4 yield than digestion of spent mushroom only [99].

The ratio of the co-digested substrates is important for a successful SS-AD. Li et al. [44] conducted a SS-AD of tomato residues, corn stover, and dairy manure with eight mixing ratios. The authors reported that a mixing ratio of tomato residues, corn stover, and dairy manure at 13:33:54 (TS based) achieved the highest CH_4 yield, while digestion of tomato residues failed due to ammonia inhibition. Similarly, co-digestion of food waste with distiller's grains under four ratios (1:4, 1:6, 1:8, 1:10) showed that food waste vs. distiller's grains ratio at 1:8 resulted in the highest CH_4 yield [98].

6.3 Additives

Various additives have been used to supplement AD systems to improve digestion performance [102]. Biochar, a charcoal-like product of incomplete combustion (pyrolysis) of organic materials, has been used as an additive in AD with multiple functions. In a study of chicken manure AD, Liang et al. [103] found that adding biochar reduced the lag phase by 41% and increased CH_4 production rate by 18% with reduced H_2S. In another study of AD of sludge amended with biochar, average CH_4 content in biogas was up to 92.3%, corresponding to a CO_2 sequestration by 66.2% [104]. A biochar addition also enhanced process stability through increasing the alkalinity and alleviated free ammonia inhibition [104]. Qin et al. [105] used magnetic biochar (a composite of biochar and magnetic medium) as an additive in sludge AD and recorded 11.69% increase in CH_4 production. The authors attributed the enhancement to the selective enrichment of functional bacteria and methanogens absorbed on magnetic biochar.

Materials promoting direct interspecies electron transfer (DIET) are also used as additives to accelerate the conversion of organic substrate to CH_4 [106]. For instance, carbon cloth and granular activated carbon were used to stimulate CH_4 production in AD of dog food, tolerate high OLR, and recover from soured digester faster [106]. Conductive materials were also effective in stimulating the syntrophic conversion of ethanol to CH_4 in upflow anaerobic sludge blanket reactor [107]. The CH_4 production rates increased by 30–45% with the addition of conductive materials at each OLR [107].

It should be noted that although various additives have been shown to be beneficial to AD systems, few studies have been done to apply those additives to SS-AD. Further studies are needed to evaluate the technical and economic feasibility of using additive in SS-AD systems.

7 Conclusion and Perspectives

SS-AD has become a popular approach to digest organic wastes with high solid content due to its inherent advantages such as high OLR, reduced reactor size, and minimal amount of digestate generated. A variety of materials, from municipal solid wastes to agricultural residues, can be used as feedstock for SS-AD. To ensure a successful SS-AD, operation conditions such as nutrient levels, feedstock-to-inoculum ratio, pH, temperature, and mixing need to be carefully controlled. Moreover, reactor systems configured with different operation modes (batch vs. continuous; one stage vs. multiple stage) have been applied based on diverse characteristics of the feedstocks. To enhance SS-AD performance, pretreatment is needed to make lignocellulosic feedstock more amenable for microorganism to degrade. Co-digestion of different feedstocks and supplement external additives such as biochar are also effective to improve biogas production.

Further studies on SS-AD should focus on several issues in order to develop an effective commercial-scale process. First, feedstock pretreatment should be carefully selected to address the operational costs, treatment effectiveness, and inhibitors. Second, mass transfer limitation needs to be effectively overcome. Finally, understanding the microbial consortium and metabolic pathways involved in SS-AD processes is crucial to provide potential guidance to improve the digestion performance. Solving these hurdles will facilitate the application of SS-AD as a promising alternative to the traditional waste disposal process.

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