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Is the conversion of lignocellulose into feed with white rot fungi realizable? Practical problems of scale up and technology transfer

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Abstract
The bioconversion of plant residues into feed and food as well as the realization of large-scale technology belong to the most important aims of ecological and economical housekeeping of mankind. The digestibility of lignocellulose used as animal feed to rumen microorganisms is limited by its lignin content. Therefore low-cost techniques for selective removal of lignin from the lignocellulose matrix are needed. This paper reviews the factors influencing selective delignification of lignocellulosics by white-rot fungi, and discusses the prospects for the development and scale up of technology and reactors for bioconversion of lignocellulosics into feed.

Keywords: lignocellulose, agriculture waste, white rot fungi, solid state fermentation, animal feed

The rumen of certain domestic and wild animals, modified during evolution, is an "ideal" fermentor, digesting cellulose and lignin containing plant residues -lignocellulosics- (Hungate, 1975; Prins and Clarke, 1979; Stewart, 1981). Digestion proceeds discontinuously by anaerobic bacteria, fungi and protozoa. Cellulose and hemicellulose (ca. 40%) are partially metabolized during a relatively short time, 2-3 days. Increase of lignin content in plant materials used as feed correlates with decrease of digestibility for rumen microorganisms. In order to increase the digestibility of lignocellulosics, physical, chemical and biological methods of delignification can be used. The principle of these methods is the splitting of the cellulose-lignin complex by extraction or decomposition of lignin. Proposed process of biological upgrading of lignocellulosics into animal feed should be characterized by marked lignin decomposition and liberation of nutrients from the lignocellulose-matrix with contemporary accumulation of digestible substances. The main constraints in optimizing biological upgrading of lignocellulosics into feed are the identification of appropriate microbial species, the characterization and elucidation of factors controlling selective delignification by microorganisms and the development of an appropriate technology to achieve a cheap large-scale process.
Financial support and scientific interest in the last decades were concentrated mainly on enzymatic studies connected with lignin and lignocellulose degradation. From academic point of view, these studies are fascinating, but did not improve the practical realization of a process for selective removal of lignin. In the history of mankind, bread, beer, vine and other fermented foodstuff were produced for a thousand of years without any knowledge about the existence of enzymes.

Ligninolytic microorganisms are mainly wood inhabiting fungi. They are able to colonize different plant residues (Zadrazil, 1976a, 1979) and increase the digestibility of the substrate (Schánel et al., 1966; Herzig et al., 1968; Kirk and Moore, 1972). The ideal microorganism for upgrading of lignocellulosics into animal feed should combine high ligninolytic capability with low degradation of cellulose and hemicellulose. The growth properties of cellulase-less mutants of three white rot fungi, with the aim of selective lignin degradation, were studied by Eriksson et al. (1980).

Primary problems of scale up of delignification process using white rot fungi are due to a lack of knowledge about the factors regulating fungal development and lignin degradation in solid state fermentation. The process needs to be cheap and simple by using low cost technology. Similar processes are suitable to convert plant residues into human food by the cultivation of edible fungi (*Pleurotus* spp., *Stopharia rugosoannulata*, *Volvariella volvacea* etc.).

Factors influencing selective delignification and *in vitro* digestibility

Influence of fungal species

The influence of the fungal species on the decomposition of wheat straw and the *in vitro* digestibility and decomposition of lignin is comprehensively discussed by Zadrazil (1985) and Capelari and Zadrazil (1997). Tested fungi can be devided into four groups. Fungi in the first group decompose the lignocellulose substrate without lignin degradation (brown rot fungi). This results in lower *in vitro* digestibility compared to the untreated substrate. Examples are *Agrocybe aegerita* and *Flammulina velutipes*. Similar results can be obtained by the cultivation of low fungi, bacteria or yeasts on cereal straw.
The second group includes fungi, which decompose lignin very well, but other substrate components are decomposed only partially (white rot fungi). This results in increasing *in vitro* digestibility. Examples are *Abortiporus biennis*, *Dichomitus squalens*, *Pleurotus eryngii*, *Pleurotus sajor-caju* and *Stropharia rugosoannulata*.
The third group includes fungi, which decompose lignin and other substrate components, but *in vitro* digestibility decreases. This may be due to toxicity for the rumen microorganisms of substrate extracts that are used for the determination of *in vitro* digestibility.
The fourth group consists of fungi that decompose lignin and other components very rapidly and change *in vitro* digestibility only partially. One example is *Sporotrichum pulverulentum* which mineralizes ca. 3% of the straw substrate per day but does not cause any significant change in digestibility (Zadrazil and Brunnert, 1982; Zadrazil, 1985).

Influence of composition and quality of the substrate

Different substrates, including beech sawdust, rape straw, sunflower straw, reed straw and rice husks, were subjected to 60 days of solid state fermentation with different fungi (Zadrazil, 1980). It was found that the speed of decomposition of organic matter, the lignin decomposition and the *in vitro* digestibility were strongly dependent on fungal species and kind of plant waste substrate. *Pleurotus* sp. florida, *Pleurotus cornucopiae* and *Stropharia rugosoannulata* showed good lignin decomposition and increased the *in vitro* digestibility of all substrates except the rice husks. This effect is probably caused by the high incrustation of rice husks with SiO_2.

Influence of fermentation temperature

The temperature of fermentation does not only influence the decomposition speed of the organic matter but also the sequence of decomposition of the substrate components. With all fungi tested, increasing of the temperature between 22°C and 30°C increased the decomposition speed of the organic matter (Zadrazil, 1977, 1985). A positive correlation between increasing of temperature and lignin decomposition or *in vitro* digestibility was only found for *Stropharia rugosoannulata*.

Influence of duration of fermentation

In the fungal live cycle the following periods can be distinguished: colonization of the substrate, maturation of the fungus, induction of fruiting bodies and autolysis. During the first stage of fungal growth, the content of digestible substances for ruminants decreases (Zadrazil, 1977). After colonization is finished, the *in vitro* digestibility of fungal substrate increases. Afterwards, in older substrates with relatively high content of accumulated minerals, it decreases again. Under favourable conditions, some fungi can mineralize cereal straw completely during 80-100 days of fermentation (Zadrazil, 1985).

Influence of NH_4NO_3 supplementation

Amendment of the substrate with NH_4NO_3 changes the decomposition rate and the sequence of decomposition of substrate components. *Stropharia rugosoannulata, Agrocybe aegerita* and *Pleurotus* sp. florida were stimulated during decomposition of the substrate by the addition of lower NH_4NO_3 concentrations, while *Pleurotus eryngii* was inhibited by these. The lignin decomposition rate was relatively unchanged by NH_4NO_3 amendment. However, a decrease of *in vitro* digestibility of substrate mixture was observed with all fungi when substrate received higher concentrations of NH_4NO_3 (Zadrazil and Brunnert, 1982)

Influence of the water content of the substrate

Zadrazil and Brunnert (1981, 1982) cultivated different fungi on substrates with varying water contents (between 50% to 85%). The fungi tested showed specific growth optima. With both the lowest and the highest water content, the decomposition rate of the total organic matter decreased, as did decomposition of lignin and accumulation of digestible substances.

Influence of the composition of the gaseous phase

For large-scale process the composition of gaseous phase is proposed to be the key-factor. Kamra and Zadrazil (1985, 1986) found the losses of organic matter and lignin after fermentation of wheat straw with *Pleurotus sajor-caju* to be highest in a 100% oxygen atmosphere. Lignin was degraded at a much lower rate with less than 20% oxygen in the atmosphere. The increase in *in vitro* digestibility was highest in pure oxygen, followed by that in the air atmosphere. CO_2 at 1-10% positively influenced the increase in digestibility, but at higher concentration the digestibility was reduced.
Zadrazil (1975) found a positive influence of low CO_2 concentration on the mycelial growth and on the digestibility of wheat straw. Cultivation under 10% of CO_2 in the gaseous phase in the case of *Pleurotus eryngii* and 20% in the case of *P. sajor-caju* favoured increase of digestibility and lignin loss more than of either air or oxygen.

Before we will be able to understand the role of CO_2 in increasing the digestibility of wheat straw completely, more detailed studies are needed in which the substrate is in closer contact with the gaseous phase. The influence of the gaseous phase composition is restricted by the surface of the substrate which is the contact aera with the gaseous phase. The composition of the gaseous phase in and near the substrate is different from that provided from outside because of fungal respiration and metabolism (Zadrazil and Kamra, 1989). Gaseous metabolites of fungal degradation of straw have a strong influence on mineralization of organic matter, loss of lignin and *in vitro* digestibility (Zadrazil et al., 1991). The influence on the composition of the substrate after different treatments with gaseous metabolites is summarized by Buta et al. (1989) and Chiavari et al. (1989).

The above investigations can be summarized as follows:

1. The *in vitro* digestibility of fungal substrates decreases at the beginning of colonization by white rot fungi and increases afterwards (Zadrazil, 1977; Zadrazil and Brunnert, 1982). During incubation the content of soluble substances (partly sugars) increases (Zadrazil, 1976b; Lindenfelser et al., 1979).
2. The increase in digestibility depends on the fungal species (Zadrazil, 1979), the cultivation time and temperature and on the preparation, water content, bulk density and composition of the substrate (Zadrazil and Brunnert, 1981, 1982).
3. The *in vitro* digestibility of lignocellulose treated with white rot fungi is decreased by addition of inorganic nitrogen (Zadrazil and Brunnert, 1980).
4. The pattern of lignocellulose degradation by white rot fungi is influenced by the concentration of carbon dioxide, oxygen and fungal metabolites in the gaseous phase (Zadrazil et al., 1991).

A pilot reactor for solid state fermentation in large scale

Above examples and the analysis of "Palo podrido" samples (Phillippi, 1893; Knoche et al., 1929; Zadrazil et al., 1982) clearly show that use of white rot fungi for upgrading lignocellulosics into feed is possible, at least on a laboratory scale and in natural processes. On the other hand, only little is known about the large-scale process. Solid state fermentation of lignocellulosics in deep layers might be proposed (Schuchardt and Zadrazil, 1982, 1988). Since 1985 at the Institute of Soil Biology, FAL Braunschweig, a SSF- reactor, serving as a model for large-scale technology, was tested (Zadrazil et al., 1990a, 1990b). A similar process is used for the production of substrates for white mushrooms (Francescutti, 1972; Bech, 1977; Overstijns, 1981).

Definition of solid state fermentation

Solid state fermentation (SSF) can be defined as a process, in which solid substrates are decomposed by known pure or mixed cultures of microorganisms (mainly fungi, which can grow on and through the substrate) under controlled conditions, with the aim of producing a high quality standardized product (different from composting). The substrate, a mixture of different particles, is characterized by a relatively low water content. Since much of the water is chemically or physically bound to the substrate, physical properties, e.g. porosity and density, are uniform. The substrate is not mixed or moved during the process (Zadrazil et al., 1990a, 1990b).

Applications of solid state fermentation

Recently many different reactors for solid state fermentation have been designed, developed and constructed. Some are used in Koji process for production of soybean sauce, or in the production of substrates for the cultivation of edible fungi, for example *Agaricus bisporus* (Francescutti, 1972; Gerrits, 1988) and *Pleurotus* spp. (Schuchardt and Zadrazil, 1982, 1988). Another proposed use for these fungal substrates is the conversion of lignocellulosics into animal feed (Laukevics et al., 1984; Matteau and Bone, 1980; Weiland, 1988; Zadrazil, 1977, 1980, 1985) and chemical feedstocks (Hatakka and Pirhonen, 1985) as well as biological pulping (Kirk et al., 1980; Eriksson, 1986; Eckstein, 1985). Prepared substrates can also be used in environmental control as biofilters (Hüttermann et al., 1988) or for decontamination of xenobiotics in soil and waste materials (Hüttermann et al., 1988, 1989).

Principle of the process

The pretreated substrate is filled into the reactor in 1.5-2.0 m deep layers and incubated by percolation of the gas phase through the substrate. The temperature in the substrate is indirectly controlled by conditioned, percolated gas. In the cultivation of *Agaricus* spp., fresh air is added in order to cool the substrate. In contrast to the production of wood decaying fungi, high carbon dioxide and low oxygen concentrations are required in the period of colonization (Zadrazil, 1975).

Design of the reactor

The fundamental design of a small-scale reactor and its control were described by Schuchardt and Zadrazil (1982, 1988). The reactor used in the Institute for Soil Biology, FAL Braunschweig, is made of polyurethane foam sandwich panels that are covered on both sides with polyester board. The filling height is approximately 2.0 m, the internal width 2.3 m and the depth 2.0 m. This results in a net filling volume of 9.2 m^3 equivalent to 1.5 t of straw or 3.0 t wood chip substrate. Two similarly insulated swing doors are situated at the front of the reactor, the width of the doors being the width of the reactor itself. Inside the reactor there is a raised slatted floor covered firstly with a gliding net and then with a drag net.
Two reactors of the same shape and construction are used. One reactor is used for substrate pretreatment and the second one for substrate colonization (Zadrazil et al., 1990a, 1990b, 1996; Zadrazil, 1997). On moving the substrate from one reactor to the other reactor, the substrate is inoculated.
The substrate is filled into the reactor using a special designed machine which deposits it on the drag net. A removable front panel keeps the substrate from falling out of the container during filling. The substrate is removed from the container by attaching the drag net to a winch. The substrate is loosened as it is pulled through a set of toothed bars before falling onto the elevator. Then it is filled into another reactor either for incubation or for treatment of the completely colonized substrate. This translocation from one reactor into the other decreases the bulk density of the substrate and reduces the stream resistance of the gas phase by the substrate. Stream resistance could be a parameter for fungal growth activity.

Air-conditioning

Fungal growth and heat exchange from the substrate is controlled by re-circulating the air within the reactor. During the incubation of *Agaricus bisporus* fresh air is added into the reactor in large quantities (Francescutti, 1972). For the cultivation of *Pleurotus* spp. and other

wood decaying fungi the gaseous phase is recycled and gas composition is controlled by monitoring CO_2 and O_2 concentrations (Schuchardt and Zadrazil, 1982, 1988). To control and reduce the growth of competitive microorganisms, CO_2 could be added at the beginning of the fermentation process.
The reactor has an air-conditioning system installed on the roof or in the gas-tubes. A system of aluminium air ducts, with supply duct below and return ducts in the roof, ventilates the substrate from below. The total quantity of circulated air can be varied by changing the speed of electronically controlled fans. A centrifugal type fan was chosen to ensure that the various processes could be controlled adequately. It has a capacity of 800 m^3/h at a static pressure of 1200 Pa, or approximately 200-500 m^3 per ton substrate and hour (depending on its specific volume weight). The most suitable fans for this purpose are of the centrifugal type, with backward curved blades, as their capacity varies only slightly with decreasing resistance.
In order to humidify the air and raise the substrate temperature for pretreatment of substrate (e.g. pasteurization), a steam injection pipe is installed under the slatted floor. Steam injection is controlled by a 2-way valve operated by a servomotor. Heat exchange takes place by cooled refrigeration in the air-circulation system controlled by a servomotor driven 4-way valve. The lowest temperature of cooling liquid can be -15°C.
Fresh air, oxygen or carbon dioxide can be added after gas analysis by computer-controlled valves. Exhaust gases leave the fermentor through an over-pressure valve located above the substrate.

Control of gas humidity and water evaporation

The humidity of gaseous phase was controlled by hygrometers situated in different parts of the reactor. The air humidity fluctuates between 95 and 100%.
Saturation deficit of water in the gaseous phase increases during the penetration of gas through the substrate. The gaseous phase has a lower temperature than the substrate and water evaporates. From the circulating gases, water condenses on the cooling equipment and within cold areas of substrate and reaches 100 % relative humidity again. Evaporation of water in circulating gases was measured by water loss from a 20 cm^2 ceramic disc (Czeratzki, 1968) placed in the space above the substrate.
Evaporation of water from the substrate and condensation on the cold parts of reactors and cooling unit is undesirable but cannot be eliminated. The translocation of water could be a measure of the technical standard of the reactor and the efficacy of the control system. All condensed water is led into the container at the base of the reactor and periodically quantified.

Temperature control

For scientific studies the ducts must be insulated to eliminate the uncontrolled loss of metabolic heat from the reactor. The rate of heat liberation during the fermentation process was measured by a flow meter installed in the cooling system of the reactor.
The temperature of the gas phase is monitored by placing 4 thermometer sensors at the inlet and outlet ducts. Temperature of substrate is measured in 4 different layers with 8 thermometer sensors and registered in 2 independent computers. Minimum and maximum limits for temperature can be adjusted. If the temperature of the air rises above or falls below the temperature limit, the heating or cooling system is activated.

Air supply and control of circulated air

The amount of air circulated in the reactor through the substrate is measured on the supply side of the fan by measuring the difference in pressure across a gauge ring. The accuracy of this apparatus is very good and air resistance is very low. The required fresh air is added with an air pump and measured with a flowmeter.

Infection by competitive microorganisms

The proposed system of SSF is based on the use of non-sterile culture conditions. Selective propagation of thermophilic and mesophilic microorganisms during substrate pretreatment supports the saprophytic colonization by the cultivated fungus. Infection of substrate was not observed during colonization of substrate under these conditions. After 14 days of preincubation, the substrate can be used for the production of edible fungi.
During the production of animal feed, colonies of *Trichoderma* sp. were observed on the surface of the substrate at the end of fermentation. This infection correlates with high substrate digestibility. Infection was frequently observed, when condition for the growth of *Pleurotus* sp. was suboptimal (e.g. temperature being too high).

Digestibility and homogenity of product

The digestibility of substrate (Tilley and Terry, 1963) on fermentation increased on average by 13.8 digestibility units. The highest increases (18.7 and 18.3 units) were found in the two layers near the substrate surface and the lowest (7.0 units) on the bottom layer (Zadrazil et al., 1990a, 1990b).
After incubation, the substrate was also used for production of edible fungi. Colonized substrate was placed into a container for fructification. The yield of fruit bodies was satisfactory and was comparable to that obtained with other cultivation systems.

Conclusions

Still insufficient knowledge exists about biological delignification of lignocellulosics in solid state fermentation and about suitable reactors for this process. More basic and applied research is needed, before this technology can be transferred in practical feed production and other areas of biotechnology (e.g. biopulping). It is supposed that for each organism special strategies for the design of the reactor and for the control of the process need to be developed. Based on the studies reported here it can be conclude that there is a particular need for further research on the following aspects:

1 Development and testing of new designs and constructions of SSF reactors.
2. Development of strategies for process control with different fungi and plant residues.
3. Development of equipment and sensors for the control of the SSF process.
   3.1 Control of the air speed in different parts of reactors.
   3.2 Control of air humidity.
   3.3 Control of water evaporation from substrate.
   3.4 Control of water translocation.
4. Verification of results in long time repeated experiments in laboratory and pilot-scale reactors.
5. Comparative economic studies with other lignocellulose-upgrading processes.
6. Control of feed values of upgraded lignocellulosics in long-time feed experiments.

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