Ultrastructure of a Thermotolerant Basidiomycete Possibly Suitable for Production of Food Protein

BENG T. HOFSTEN AND ANGELICA V. HOFSTEN

Institute of Biochemistry and Institute of Physiological Botany, University of Uppsala, Uppsala, Sweden

Received for publication 24 January 1974

The imperfect cellulolytic fungus Sporotrichum pulverulentum, which is commonly found growing in wood-chip piles, was grown in submerged culture on wheat shorts and other cereal flours. These substrates were broken down in 1 to 4 days at 30 to 40 C, and the mycelial mass was easily harvested by filtration. Scanning electron micrographs of hyphae in mycelial pellets are presented, and thin sections of conidia and hyphae were studied in a transmission electron microscope. Dolipores in septa of hyphae were observed, and cell walls are shown to be lamellar, which is characteristic of the Basidiomycetes. Actively growing hyphae are full of cytoplasm with numerous mitochondria, whereas old mycelial pellets contain highly vacuolated and almost empty cells.

Large quantities of fruit bodies (sporophores) of many kinds of Basidiomycetes have long been eaten because they are nutritious and good tasting. Some higher fungi can be grown in submerged culture, but most of them grow slowly in conventional fermentors and only a few have been used for industrial production of food products (12). Other filamentous fungi, classified either as Ascomycetes or Fungi Imperfecti, are presently being investigated as unconventional sources of protein (13), and commercial production of "mycoprotein" by cultivation of a Fusarium sp. has recently been started (9).

Before large-scale production of fungal biomass for human consumption is considered, it is necessary to prove that the product is nontoxic. Information on the systematic position of the organism studied is then useful. It is also important to determine how culture conditions influence the chemical composition and morphology of the organism. The protein content may vary considerably depending on culture conditions, but the nucleic acid content of fungi is usually lower than that of many bacteria, yeasts, and algae (13). Single-cell organisms may be easier to grow in submerged culture than fungi, which have a filamentous or pellet form of growth, but the latter can often be harvested by simple filtration.

We have for some years studied the microbial degradation of cellulose and related polysaccharides. As part of this project, we have investigated the possibility of growing cellulolytic microorganisms on flours and meals of various cereals and other seeds. One such material is wheat bran, which contains 15 to 17% protein and about 70% carbohydrates, about 80% of which is cellulose and hemicellulose. Very large quantities of bran and other milling fractions containing bran are at present either used only as low-quality animal feed or wasted. The same applies to such products as rice bran, and it would be important if they could be converted to products of higher nutritional value. We have found that a thermotolerant, cellulolytic fungus can grow on various cereal flours, and preliminary experiments indicate that the mycelium has a high nutritional value. The organism we used is a wood-destroying, imperfect fungus that was isolated from a pile of wood chips by Nilsson (6). He found that it caused typical white-rot decay, and the organism was first classified as a Chrysosporium sp., but it has now been found (J. A. Stalpers, personal communication) to be identical with a fungus described as Sporotrichum pulverulentum by Novobranova (7). Its cellulolytic enzymes have been studied by Eriksson and Rzedowski (3), who purified several exo- and endoglucanases and other carbohydrases from cell-free fluids of submerged cultures of the fungus.

This report describes some features of the ultrastructure of S. pulverulentum and effects
of cultivation time on its cytoplasm. Studies on the effect of culture conditions on its growth morphology and chemical composition and the results of pilot-scale cultivation experiments are presented elsewhere.

MATERIAL AND METHODS

Fungal strain. The strain of *S. pulverulentum* that we studied was isolated in 1964 by Thomas Nilsson, College of Forestry, Stockholm, where it is maintained in the culture collection as strain P 127. It has also been deposited at Centralbureau voor Schimmelmicroscopie, Baarn, The Netherlands, where it has the number CBS 671.71.

Cultivation methods. Stock cultures were maintained on malt extract agar (Difco), on which large numbers of white conidia are formed.

Submerged cultures were grown in the following mineral salts medium: KH₂PO₄ (4 g), K₂HPO₄ (1 g), (NH₄)₂SO₄ (3 g), and MgSO₄·7H₂O (0.2 g per liter of tap water). Carbon sources were added at a concentration of 1 to 2% (wt/vol), and the pH was adjusted to 6 before autoclaving.

Scanning electron microscopy. Freeze-dried cell material was coated with evaporated gold before examination in a JSM-U3 scanning electron microscope operating at 25 kV.

Transmission electron microscopy. Mycelial pellets from submerged cultures and pieces of agar containing hyphae were prefixed for 24 h in 2.5% glutaraldehyde buffered with Veronal-acetate buffer, pH 7. The material was further fixed in 2% potassium permanganate in water (4). After dehydration in a graded ethanol series, the material was impregnated with the plastic resin Epon 812, and this was polymerized at 60 C. Thin sections were cut with a diamond knife on an LKB ultramicrotome, and sections were post-stained with 2% aqueous uranyl acetate for 20 min at 60 C and with 6% aqueous lead citrate for 2 min at room temperature. Specimens were examined in a Jeol 100 B electron microscope operating with a double condenser at 60 kV.

RESULTS

Growth in liquid cultures. Conidia from agar cultures were used to inoculate Fernbach flasks containing a shallow layer of mineral salts medium with 1 or 2% wheat shorts. The cultures were incubated on a rotary shaker (150 rpm) at 30, 35, or 40 C. The spores germinated and formed small hyphal aggregates after about a day. In this medium and under these culture conditions, the small aggregates grew out into spherical pellets which increased in size during the following day with a concomitant decrease in pH to between 4 and 5. The pellets could easily be filtered off on a 30-mesh sieve giving an almost clear, slightly yellow filtrate which had a pleasant smell reminiscent of apples. The maximal growth temperature was between 40 and 45 C, but the fungus cannot be called thermophilic because it also grew below 25 C.

Other milling fractions of wheat could be converted to fungal biomass at rates which depended on the fineness of the flour. It took 4 to 5 days at 35 C to degrade coarse wheat bran, whereas fine flours of rye and barley were degraded in a couple of days. Defatted rice bran, which consists of very fine particles, supported rapid growth and was completely degraded in about 2 days.

Figure 1 illustrates a sample of freeze-dried mycelial pellets obtained on wheat shorts. Some small bran fragments were undegraded at the time of harvesting, but these could be washed away through a 30-mesh sieve. Growth on whole wheat flour gave smaller (1 to 2 mm), irregular hyphal aggregates, whereas very smooth, 4- to 5-mm pellets were obtained on wheat bran.

Electron microscopy. Figure 2 is a scanning electron micrograph of the interior of a mycelial pellet harvested from a 3-day-old culture on wheat bran. The mycelium is highly branched, and it can be seen at higher magnification (Fig. 3) that many of the hyphae have collapsed to flat, fiber-like structures during the freeze-drying.

Few conidia are formed in liquid cultures unless these are incubated for a long time or left standing at room temperature for some days. Figure 4 is a thin section of a young conidium attached to a hypha by means of a stalk cell which contains a supporting structure near the conidium. A cap-like structure can also be seen on top of the conidium, and its cell wall is still rather thin. When observed in the light microscope, most conidia are spherical, but some of them are dumbbell shaped (Fig. 5). This mature conidium has a thick, multilayered wall measuring almost 1 μm, and it contains several nuclei and numerous mitochondria.

Hyphae have thinner walls than the conidia, and old cells are often highly vacuolated. Figure 6 shows a thin section of a young hypha grown on malt agar. There is a distinct dolipore between the two cells and a peculiar membranous structure near the septum. The insert in Fig. 6 shows that the hyphal wall has a distinct lamellar structure, and pictures of old hyphae showed that these lamellae occasionally become partly separated from each other.

Samples of small mycelial aggregates from 1 to 2 days old submerged on wheat shorts cultures contained a large proportion of hyphae which had an appearance similar to that of the hypha shown in Fig. 6, but very few of the septa had typical dolipores. Cultures which had been
Fig. 1. Freeze-dried mycelial pellets of *S. pulverulentum*.

Fig. 2. Scanning electron micrograph of hyphae in a mycelial pellet.
grown for more than 3 days on wheat bran contained 4- to 5-mm pellets in which the cells were more or less autolyzed. Figure 7 shows a thin section of some hyphae in a pellet grown on wheat bran for 5 days.

DISCUSSION

The increasing global demand for high-quality proteins for human food and animal feed has stimulated much work on large-scale production of microbial cells. Single-cell organisms are often easier to grow in submerged culture than filamentous fungi, but they are more difficult to harvest, and many of them contain so much nucleic acid that they cannot be used for human consumption. Many higher fungi are already accepted as a source of food in many parts of the world, but most of them grow slowly at rather low temperatures. Worgan (13) has recently discussed the relative merits of different substrates and general problems in the production of microbial protein. Cellulolytic fungi have earlier been tried for the conversion of paper and various agricultural wastes to animal feed (1, 8, 10), but many cellulolytic organisms grow very slowly or not at all on lignified plant material. Expensive pretreatments are therefore necessary in order to make such raw materials degradable. Some of the fungi tested, such as Trichoderma viride, Aspergillus fumigatus, and various Fusarium sp., may also produce mycotoxins under certain conditions. The fungus that we studied has some properties which suggest that it may become an interesting new source of protein. It grows rapidly at relatively high temperatures and can degrade a variety of cheap carbohydrates such as cellulose, hemicellulose, and starch, and it can even grow on lignified tissues. Its systematic position remains somewhat uncertain as long as it must be classified among the Fungi Imperfecti, but our electron micrographs show...
Fig. 6. Thin section of a hypha growing on malt extract agar. Note the dolipore (P) between the two cells and the membranous structure (MS) near the septum. The insert shows the lamellar structure of the wall at higher magnification.

Fig. 7. Thin section of autolyzed hyphae in a pellet grown on wheat bran for 5 days.
that it must be an imperfect conidial stage of a basidiomycete. The presence of the characteristic dolipores in the septa of hyphal cells is thus a characteristic feature of Basidiomycetes (2). The lamellar structure of the hyphal walls is also considered to be typical of this group of fungi (5). Furthermore, only Basidiomycetes are known to cause white-rot attack on wood.

It is possible that S. pulverulentum is closely related to the higher fungi of the family Polyporaceae, of which many are eaten. We have as yet only carried out preliminary feeding experiments with mycelium grown on wheat shorts, but no harmful effects were noticed when the fungus was grown as sole source of protein to young rats, and their rate of growth was normal for over 3 weeks. More extensive feeding tests must, of course, be carried out to prove that the fungus is wholesome.

Brans and other milling fractions of cereals contain cellulose, hemicellulose, and starch in varying proportions, and they are interesting raw materials for mycoprotein production. They are available in very large quantities and can easily be stored. Several technical problems must be solved before a fungus can be grown on a large scale on such partly insoluble substrates. Many fungi grow either in filamentous or pellet form, depending on the nature of the inoculum, composition of the medium, or physical conditions (11). It is important to be able to control these factors so that a maximal protein content is obtained in the mycelium. Our electron microscope studies show that large pellets of S. pulverulentum contain a high proportion of partly autolysed cells, and it has been found that the protein content of these cells is only about 20%. It is therefore obviously desirable to grow the organism under conditions which favor the formation of small hyphal aggregates.

**ACKNOWLEDGMENTS**

We thank Karl-Erik Eriksson for providing us with a culture of the fungus and Thomas Nilsson for valuable discussions. We are very grateful to J. A. von Arx and J. A. Stalpers, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, for help with the identification of the fungus and comments on our manuscript. We also thank Leif Tibell for taking the scanning electron micrographs and Ingvors Andersson and Anette Eriksson for skillful technical assistance.

This work was supported by a grant from the Swedish Board for Technical Development.

**LITERATURE CITED**

1. Chahal, D. S., G. D. Munshi, and S. P. S. Cheema. 1969. Fungal protein production by Rhizoctonia solani when grown on cellulose as the sole source of organic carbon. Proc. Nat. Acad. Sci. India 39(3):287–291.
2. Donk, M. A. 1973. The Heterobasidiomycetes: a reconnaissance. III. How to recognize a Basidiomycete? Proc. Kon. Ned. Akad. Wetensch. Ser. C 76:1–22.
3. Eriksson, K. E., and W. Rzedowski. 1969. Extracellular enzyme system utilized by the fungus Chrysosporium lignorum for the breakdown of cellulose. Arch. Biochem. Biophys. 129:690–695.
4. Hedenskog, G., and A. V. Hofsten. 1970. The ultrastructure of Spirulina platensis—a new source of microbial protein. Physiol. Plant. 23:209–216.
5. Kreger-Van Rij, N. J. W., and M. Veenhuis. 1971. J. Gen. Microbiol. 68:87–95.
6. Nilsson, T. 1965. Mikroorganismer i flisstackar. Svensk Papperstidn. 68:495–499.
7. Novobranova, T. I. 1972. Species novae fungorum imperfectorum e regione Alma-Ataensi. Novosti Sist. Nizhchik Rastenii 9:180.
8. Rogers, C. J., E. Coleman, D. F. Spino, T. C. Purcell, and P. V. Scarpino. 1972. Production of fungal protein from cellulose and waste cellulosics. Environ. Sci. Technol. 6:715–719.
9. Spicer, A. 1973. Proteins from carbohydrates. Chem. Brit. 9:100–103.
10. Toyama, N., and K. Ogawa. 1972. Utilization of cellulosic wastes by Trichoderma viride, p. 743–757. In G. Terui (ed.), Fermentation technology today.
11. Whitaker, A., and P. A. Long. 1973. Fungal pelleting. Proc. Biochem. 8:27–31.
12. Worgan, J. T. 1968. Culture of the higher fungi. Progr. Ind. Microbiol. 8:73–139.
13. Worgan, J. T. 1973. Protein production by microorganisms from carbohydrate substrates, p. 339–361. In J. G. W. Jones (ed.), The biological efficiency of protein production. Cambridge University Press, New York.