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Thermophilic Fungi: Their Physiology and Enzymes†

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INTRODUCTION

Among the eukaryotic organisms, only a few species of fungi have the ability to thrive at temperatures between 45 and 55°C. Such fungi comprise thermophilic and thermotolerant forms, which are arbitrarily distinguished on the basis of their minimum and maximum temperature of growth (63): the thermophilic fungi have a growth temperature minimum at or above 20°C and a growth temperature maximum at or above 50°C, and the thermotolerant forms have a temperature range of growth from below 20 to ~55°C. Thermophily in fungi is not as extreme as in eubacteria or archaea, some species of which are able to grow near or above 100°C in thermal springs, solfatara fields, or hydrothermal vents (36, 45). Perhaps because of their moderate degree of thermophily and because their habitats are not exotic, thermophilic fungi have not received much publicity and attention. However, considering that the vast majority of eukaryotes cannot survive prolonged exposure to temperatures

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TABLE 1. Taxonomic status and cardinal temperatures of thermophilic fungi[a]

Fungus (present nomenclature)	Other names	T_{Opt} (°C)	T_{max} (°C)
Canariomyces thermophila Guarro & Samson		45	
Chaetomium mesopotamicum Abdullah & Zora		45	52
Chaetomium thermophile La Touche	*C. thermophilum*, *C. thermophilium*	45–55	58–61
Coonemeria aegyptiaca (Ueda & Udagawa) Mouchacca	*Thermoascus aegyptiacus*, *Paecilomyces aegyptiaca*	40	55
Coonemeria crustacea (Apinis & Chesters) Mouchacca	*Thermoascus crustaceus*, *Dactylomyces crustaceus*, *Paecilomyces crustaceus*	40	<60
Coonemeria verrucosa (Yaguchi, Someya et Udagawa) Mouchacca	*Thermoascus crustaceus*	30–40	55
Corynascus thermophilus (Fergus & Sinden) van Klopotek	*Thielavia thermophila*, *Myceliophthora fergusii*, *Chrysosporium fergusii*	50	60
Dactylomyces thermophilus Sopp	*Thermoascus thermophilus*, *Thermoascus aurantiacus* (misapplied name)	40–45	
Malbranchea cinnamomea (Libert) van Oorschot & de Hoog	*Trichothecium cinnamomeum*, *Thermoidium sulfureum*, *Malbranchea pulchella* var. *sulfurea*	45	57
Melanocarpus albomyces (Cooney & Emerson) von Arx	*Myriococcum albomyces*, *Thielavia albomyces*	45	57
Melanocarpus thermophilus (Abdullah & Al-Bader) Guarro, Abdullah & Al-Bader	*Thielavia minuta* var. *thermophila*	35	50
Myceliophthora hinnulea Awao & Udagawa		40–45	>50
Myceliophthora thermophila (Apinis) van Oorschot	*Sporotrichum thermophilum/thermophile*, *Chrysosporium thermophilum*, *Myceliophthora indica*, *Corynascus heterothallicus*	45–50	55
Myriococcum thermophilum (Fergus) van der Aa		45	53
Paecilomyces varioti Bainier[b]		50	55
Rhizomucor miehei (Cooney & Emerson) Schipper	*Mucor miehei*	35–45	57
Rhizomucor pusillus (Lindt) Schipper	*Mucor pusillus*	35–45	55
Scytalidium thermophilum (Cooney & Emerson) Austwick	*Torula thermophila*, *Humicola grisea* var. *thermoidea*, *Humicola insolens*	40	58
Stilbella thermophila Fergus		35–50	55
Talaromyces byssochlamydioides Stolk & Samson	*Paecilomyces byssochlamydioides*	40–45	>50
Talaromyces emersonii	*Geosmithia emersonii*; *Talaromyces duponti* and *Penicillium duponti* (misapplied names)	40–45	55
Talaromyces thermophilus	*Penicillium duponti*	45–50	60
Thermoascus aurantiacus	*Thermoascus aurantiacus* sensu Cooney & Emerson (misapplied name)	49–52	61
Thermomyces ibadanensis Apinis & Eggins		42–47	61
Thermomyces lanuginosus Tsiklinskaya	*Humicola lanuginosa*	45–50	60
Thermomyces stellatus (Bunce) Apinis	*Humicola stellata*	40	50
Thielavia australiensis Tansey & Jack		35–40	50
Thielavia pingtungia Chen K.-Y. & Chen Z.-C.		40	>50
Thielavia terrestris (Apinis) Malloch & Cain	*Allescheria terrestris*, *Acremonium alabamensis*	40–45	52

[a] Temperature data are from various sources and should be regarded as approximate. Because of uncertainty about the minimal temperature of growth (see text), this is not given. T_{Opt}, optimal temperature; T_{max}, maximum temperature.
[b] Confusion exists regarding its designation as a thermophilic fungus.

above 40 to 45°C (8), the ability of some 30 species, out of approximately 50,000 recorded fungal species, to breach the upper temperature limit of eukaryotes is a phenomenon that deserves elucidation. Moreover, this group of fungi provides scientists with valuable experimental material for investigations of the mechanisms which, although allowing their growth at moderately high temperatures, limit it beyond 60 to 62°C (243).

Thermophilic fungi are the chief components of the microflora that develops in heaped masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter wherein the warm, humid, and aerobic environment provides the basic conditions for their development (10, 172). They constitute a heterogeneous physiological group of various genera in the Phycomycetes, Ascomycetes, Fungi Imperfecti, and Mycelia Sterilia (182).

NOMENCLATURE

While reviewing the literature, we faced difficulties on account of the confusing nomenclature of thermophilic fungi. The confusion is due to several reasons. Since the early taxonomic literature is scattered and is often in languages other than English, it was difficult to ascertain the priority associated with the names of a species. As a result, some species have been described repeatedly under different names. As and when the earlier names were discovered, the fundamental rule of priority was applied and the names of the taxa were changed from time to time. For example, the ubiquitous fungus *Thermomyces lanuginosus*, which has been frequently used in experimental studies, has several synonyms (Table 1). Even in recent times, in several papers this fungus has been referred to by its earlier name, *Humicola lanuginosa*. Another source of confusion is the practice of interchangeably using the names of the asexual (anamorph) and the sexual (teleomorph) stages of the same fungus. For example, *Sporotrichum* (*Chrysosporium*) *thermophile* and *Myceliophthora thermophila* are, respectively, the anamorph and teleomorph stages of the same fungus. This fungus is reportedly a heterothallic ascomycete (182), but the heterothallic nature of an isolate cannot be demonstrated unless a compatible strain of the opposite mating type is available. There also are instances in the literature of misidentifications of thermophilic fungi. One example is *Thermoascus*

aurantiacus, which has featured in early physiological investigations and in several recent reports dealing with enzymological studies. Some investigators have identified their isolates based on a description of *T. aurantiacus* given by Cooney and Emerson (63), who depicted this taxon as having an asexual stage, although in reality it lacks an asexual stage. Rather, the diagnosis of *T. aurantiacus* as given by Cooney and Emerson fits that of *Dactylomyces crustaceus*, which has a *Paecilomyces* asexual stage (16). Since both *T. aurantiacus* and *D. crustaceus* became a source of confusion, Mouchacca (182) proposed that the name *T. aurantiacus* be retained whereas *D. crustaceus* be renamed as *Coonemeria crustacea*. As is now known, *T. aurantiacus* is an ascomycetous fungus with a bright orange color, elliptical ascospores, and no asexual stage. Unfortunately, unless cultures used by different investigators under the name of *T. aurantiacus* are reexamined, it may not be possible to determine which fungus was actually used.

To nonmycologists, confusion has also resulted from the merging of what, for many years, had been regarded as different taxa. For example, several scientific papers deal with polysaccharide-degrading enzymes and trehalase of *Humicola insolens*, *H. grisea* var. *thermoidea*, or *Torula thermophila*, fungi which are commonly found in mushroom composts and in soil. All these are now thought to represent one single variable species, *Scytalidium thermophilum* (236). Supposing a biologist wishes to follow on the report of an interesting enzyme found in *H. grisea* var. *thermoidea*, he or she may be at a loss to reproduce the observations unless the original culture used by the author is available. Finally, in some cases, the specific epithet *thermophilum* (or variants thereof) has been used without adhering to the proposed definition of a thermophilic fungus. To name a few, these cases include *Achaetomium thermophilum*, *Sordaria thermophila*, or *Gilmaniella thermophila*, which are thermotolerant rather than thermophilic species (however, the dividing line between the two types of fungi is thin). Mouchacca (182) has attempted to remedy the confusion that had arisen by performing a critical analysis of the nomenclature and taxonomic status of thermophilic fungi. The current names of thermophilic fungi and their synonyms are given in Table 1. It will be some times before the proposed names of thermophilic fungi are stabilized. In this paper, when the work of earlier authors is reviewed, the names of the fungi as reported in the original publications have been retained, but these should be cross-checked by reference to Table 1.

HISTORICAL BACKGROUND

The first of the known thermophilic fungi, *Mucor pusillus*, was isolated from bread and described over a century ago by Lindt (148). A little later, Tsiklinskaya discovered another thermophilic fungus, *Thermomyces lanuginosus*, growing on potato which had been inoculated with garden soil (252). Both these molds were essentially discovered as chance contaminants. The natural habitats of thermophilic fungi and the biotic conditions which favored their growth remained unknown until Hugo Miehe investigated the causes of self-heating and spontaneous combustion of damp haystacks (172). In solving the puzzle of thermogenesis of stored agricultural products, Miehe was drawn to study the microflora present therein. He was the first person to work extensively on thermophilic microorganisms. He isolated four species of thermophilic fungi from self-heating hay: *Mucor pusillus*, *Thermomyces lanuginosus*, *Thermoidium sulfureum*, and *Thermoascus aurantiacus*. He compared the heating capacities of mesophilic and thermophilic fungi (173, 174). He inoculated sterilized hay and other substrates kept inside insulated flasks with pure cultures of individual fungi and observed that the final temperature of the material depended on the maximum temperature of growth of the fungus used. He demonstrated thereby that heating of packed plant material was caused by the microorganisms present therein. Miehe explained the self-heating of hay and other plant material as follows. Initially, because of the exothermic reactions of the saprophytic, mesophilic microflora present therein, the temperature of the material rises to ~40°C. The resulting warmed environment favors the germination of spores of the thermophilic microflora, and eventually the latter outgrows the mesophilic microflora; in the process, the temperature of the mass is raised further to 60°C or even higher.

By the beginning of the 20th century, Miehe's work had led to the discovery of a small group of thermophilic fungi and to their primary habitats. Their unique thermal adaptation attracted the attention of Kurt Noack (188), who isolated thermophilic fungi from several natural substrates. He was intrigued by the fact that in addition to self-heating masses of hay and compost heaps of leaves, these fungi were present in places where temperatures conducive to their growth occur only infrequently, for example in soils of the Temperate Zone. This puzzling aspect of the ecology of thermophilic fungi provided the foundation for Noack's pioneering investigations of their physiology. Using respiration as the probe, Noack sought to determine if thermophilic fungi had an unusually high rate of respiration whereby the released metabolic heat could warm their environment, allowing them to complete their life cycle rapidly. He found, however, that the respiration of thermophilic fungi does not confer any special advantage on them.

During the Second World War, the need for finding alternate sources of rubber led to studies of the rubber-producing guayule shrub, *Parthenium argentatum*. It had been observed that the extractability and physical properties of rubber from the shrub improved when the plant material was chopped and stored in a mass before being milled. Allen and Emerson (10) demonstrated that the observed improvement from the above treatment (retting) resulted primarily from utilization and reduction in the amount of resin in crude rubber by a thermophilic microflora. From the self-heating mass of chopped guayule, Allen and Emerson isolated several species of thermophilic fungi that had temperature limits extending up to 60°C. They demonstrated that for optimal development of thermophilic fungi in the mass of material, its moisture and nutrient content were crucial. In addition, although size of the mass was an important factor for reducing the outward dissipation of heat, the mass of material had to be sufficiently porous for air to diffuse inside and allow aerobic respiration of fungi. Based on the isolates of thermophilic fungi from the retting guayule shrub and on collections of cultures from other investigators, Cooney and Emerson (63) provided taxonomic descriptions of 13 species known at that time, an account of their habitats, and the general biology of thermophilic fungi. This monograph, in English, for the first time made mycologists generally aware of the existence of thermophilic fungi. It stimulated the search for new species in order to understand their taxonomic diversity as well as to investigate their potential use as sources of commercially important enzymes. The taxonomy (182) and ecology (153, 244) of thermophilic fungi have been reviewed previously. The other important areas that have been studied in this group of fungi include their physiology and the purification and study of the functional characteristics of their enzymes. This review therefore covers the studies in these two areas from the time when experimental work on thermophilic fungi began.

PHYSIOLOGY

Growth Medium

Noack (188) grew *Thermoascus aurantiacus*, *Anixia spadicea* (*Chaetomium thermophile*?), *Mucor pusillus*, *Thermomyces lanuginosus*, and *Thermoidium sulfureum* in a glucose-salt liquid medium fortified with peptone and, often, with a decoction of hay. Until the 1980s, thermophilic fungi were thought to have complex or unusual nutritional requirements. For example, Miller et al. (176) remarked that "no defined medium could be produced in which the thermophilic fungi would grow . . . ." Rosenberg (218) reported that nearly half of the species of thermophilic and thermotolerant fungi tested required 0.01% yeast extract for growth in a solid medium. Wali et al. (260) reported that for growth in a liquid medium containing glucose and ammonium sulfate, the thermophilic fungi required a supplementation of succinic acid, a tricarboxylic acid cycle intermediate. While this observation was confirmed in our laboratory (102), we additionally demonstrated that because of the low phosphate concentration in the culture medium, the pH of the medium in the absence of the organic acid dropped to ~3.4 after 12 to 24 h and the growth ceased. Moreover, any one of the several tricarboxylic acid cycle acids tested stimulated growth, which was due to their buffering action in the medium rather than to their nutritional role: thermophilic fungi grew satisfactorily in a minimal medium if the pH of the medium was controlled between 5.5 and 7.0 by increasing the phosphate concentration in the medium, readjusting the pH by addition of an alkali, including powdered calcium carbonate as a reserve alkali, or replacing the inorganic nitrogen source with an organic nitrogen source (L-asparagine). The low pH reduces the solubility of CO_2 in the growth medium and limits its availability for assimilation by the anaplerotic enzyme pyruvate carboxylase (102). Although CO_2 is not regarded as a nutritional requirement for fungi, growth of *T. lanuginosus* was severely affected if the gas phase in the culture flask was devoid of CO_2. The concentration of CO_2 inside composts can be as high as 10 to 15% (74); therefore, it is likely that its assimilation plays nutritional and morphogenetic roles in the development of thermophilic fungi, which are the primary components of the microflora of such habitats. It is interesting that this gas has been identified as essential for the axenic culture of a rust fungus, which had long been regarded as an obligate parasite on plants (37).

Minimal Temperature for Growth

Thermophilic fungi have a widespread distribution in tropical as well as temperate regions (157). Tendler et al. (247) remarked that "the ubiquitous distribution of organisms, whose minimal temperature for growth exceeds the temperatures obtainable in the natural environment from whence they were isolated, still stands as a 'perfect crime' story in the library of biological systems." They considered whether eukaryotic thermophily is an artifact of the nutritional environment. To test this, thermophilic isolates of *Humicola*, *Thermoascus*, and *Aspergillus* were incubated in a nutritionally rich liquid medium that included glucose, mannitol, starch, Casamino Acids, yeast extract, and peptone. After 10 days at 20°C, these fungi had generated good growth, although they had failed to grow below 30°C in a sucrose-salts medium that lacked complex supplements. It was suggested that the complex materials contained a factor(s) which the organisms could not synthesize at the lower temperature. This suggestion was supported by the observation that the growth of *Talaromyces thermophilus* at a suboptimal temperature (33°C) benefited from the supplementation of culture medium with 5 μg of ergosterol per ml (264).

Furthermore, the choice of inoculum, i.e., spores versus germinated spores or mycelium, may also influence the minimal temperature of growth of thermophilic fungi. Whereas *Mucor miehei* did not grow at 25°C in submerged cultures when spores were used as the inoculum, substantial growth occurred at this temperature (nearly 64% of that at 48°C in 72 h) in as much time when pregerminated spores were used as inocula (237). Similarly, we observed that a mycelial inoculum, but not a spore inoculum, resulted in a near maximal yield of *Thermomyces lanuginosus* at 25°C; growth occurred without a perceptible lag but at a lower rate than at 50°C (Fig. 1). In *Thermoascus aurantiacus*, the lowest temperature at which the ascospores germinated was 10 to 12°C higher than that for hyphal growth (71). Presumably this may be a means of minimizing competition from the mesophilic fungi and ensuring that warm conditions would be available for yet more time until the mycelium is established for resource capture. In light of the observations that the conditions for spore germination can be more exacting than those for hyphal growth, the reported minimal temperature of growth of thermophilic fungi should be redetermined, specifying the method of inoculation and the composition of the medium used. The above observations also suggest that once the spores have been induced to germinate at high temperature, the requirement of high temperature for sustaining growth may not be critical.

Homeoviscous Adaptation

Many organisms vary the fatty acid composition of their membrane phospholipids as a function of growth temperature so that their membrane fluidity is kept constant for the optimal functioning of membrane-localized transporters and enzymes. For example, with an increase in temperature, there is an increase in the proportion of saturated fatty acids incorporated into phospholipids, whereas at lower temperature, a higher proportion of unsaturated fatty acids is incorporated. This phenomenon is called homeoviscous adaptation (229). Wright et al. (264) examined whether an inability to regulate membrane fluidity may be a reason for the high minimum temperature of growth of thermophilic fungi. They reported that when *Talaromyces thermophilus* was shifted from a high (50°C) to a low (33°C) growth temperature, the degree of unsaturation of fatty acids at the two stated temperatures remained virtually unchanged. This was thought to be the result of a metabolic limitation, presumably due to a nonfunctional fatty acid desaturase, which restricted the ability of the fungus to convert oleate to linoleate at low temperature. However, results with *T. lanuginosus* were different. In this fungus, the concentration of linoleic acid (18:2) was twofold higher at 30 than at 50°C. The degree of unsaturation of phospholipid fatty acids was 0.88 in mycelia grown at 50°C but 1.0 in the temperature-shifted cultures (from 50 to 30°C) and 1.06 in cultures grown at constant 30°C (209). A decrease in the degree of unsaturation was also observed in *Chaetomium thermophile* when it was subjected to heat shock (190). As mentioned above, some species of thermophilic fungi are capable of growth even at mesophilic temperatures. Therefore, it seems unlikely that the inability to adjust membrane fluidity is the general reason for their high minimum temperature of growth. As was reasoned for thermophilic bacteria (240), the loss of catalytic potential of one or more vital enzymes, caused by conformational changes and/or ribosomal assembly, may be an important determinant of the minimal growth temperature.

Sensitivity to Subminimal Temperatures

The need to explain the occurrence of thermophilic fungi in soil, which may warm to favorable temperatures because of

TABLE 2. Comparison of the growth parameters of some mesophilic and thermophilic fungi

Fungus	Type of fungus	Growth temp (°C)	Specific growth rate (h^{-1})	Molar growth yield (Y_G) (g/mol)	Reference
Neurospora crassa	Mesophile	30	0.37	NR[a]	7
Trichoderma reesei	Mesophile	30	0.20	72	160
Aspergillus niger	Mesophile	30	0.24	128	208
Trichoderma viride	Mesophile	30	0.16	59	208
Sporotrichum thermophile	Thermophile	50	0.23	74	208
		30	0.11	65	
Thermomyces lanuginosus	Thermophile	50	0.23	101	208
		30	0.06	87	

[a] NR, not reported.

solar radiation, but only for a transitory period, prompted Noack (188) to investigate the effects of subminimal temperatures and rewarming. He noticed that when an actively growing culture of *Thermoascus aurantiacus* was cooled to 31°C (4°C below the lower temperature limit of growth), its respiration after 2 days declined only minimally but that cultures kept at 21°C for 24 h stopped respiring. When the culture medium was rewarmed to 46°C, practically no respiration was observed. Whether the low resistance to lower temperature displayed by *T. aurantiacus* is also applicable to other thermophilic fungi is not known. However, until more information is available, it should not necessarily be assumed that mycelial cultures of thermophilic fungi can be stored under refrigeration or at subminimal temperatures without loss of viability.

Oxygen Requirement

Noack (188) recognized that during the self-heating process the environment in composts would become oxygen deficient. He therefore studied the behavior of *Thermoascus aurantiacus* subjected to anaerobiosis and found that the withdrawal of oxygen severely affected its respiration and growth. Although thermophilic fungi do not have the ability to undergo anaerobic growth (136), *Humicola insolens* was reported to grow better under anaerobic or microaerobic conditions than under aerobic conditions at elevated temperatures (116; also see reference 78). Cooney and Emerson (63) reported an interesting morphogenetic effect of anaerobiosis in *Talaromyces* (*Penicillium*) *duponti*. This thermophilic fungus formed only a conidial stage (*Penicillium*) in aerobic cultures; the sexual stage (*Talaromyces*) was initiated in agar cultures only when they were flushed with nitrogen. It would be worthwhile to study the effect of anaerobic conditions in species in which the sexual stage has not been discovered so far or is observed only infrequently.

Economic Coefficient and Growth Rate

We referred earlier to the work of Kurt Noack (188). He sought to determine whether thermophilic fungi have an exceptionally high rate of metabolism accompanied by a high rate of substrate conversion. He observed that the economic yield (grams of sugar consumed per gram of mycelial dry weight formed) of *Thermoascus aurantiacus* grown in a minimal medium at 45°C (1.89) was the same as that of the mesophilic fungus *Aspergillus niger* grown at 25°C. From this, he inferred that the overall metabolisms of the two types of fungi must be quite similar. Moreover, he estimated that on average both types of fungi converted 55% of sugar for the synthesis of fungal biomass and 45% for metabolism. Since few values of economic coefficients have been reported, we compared the molar growth yield (grams of biomass produced per mole of glucose utilized) (Y_G) of fungi. From the data available (Table 2), the average Y_G values of mesophilic and thermophilic fungi at their respective temperature optima are quite comparable (86 to 88 g/mol), suggesting that similar proportions of carbohydrate are used by both types of fungi for macromolecular synthesis. This value is close to that found by Noack for the fungal species studied by him.

Under the culture conditions used by us, some thermophilic fungi (*Thermomyces lanuginosus*, *Penicillium duponti*, *Sporotrichum thermophile*, and *Malbranchea pulchella* var. *sulfurea*) produced exceptionally homogeneous mycelial suspensions when grown in a glucose-asparagine medium in shake cultures at 50°C (102, 154, 200). This allowed the sampling of mycelia by using pipettes for quantitative measurements during their growth and facilitated the determination of growth rates, growth yields (see above), the effect of temperature-shift, and other aspects of physiology. The ranges of growth rates of thermophilic and meophilic fungi were similar (Table 2). Interestingly, although the growth of *T. lanuginosus* at a suboptimal temperature was slowed, biomass production was not affected (Fig. 1). Using CO_2 produced as an index of development of fungal biomass, Wiegant (262) observed that the exponential growth rate of the thermophilic fungus *Scytalidium thermophilum* (a common thermophilic fungus in mushroom compost) at 45°C in a liquid medium supplemented with malt and yeast extracts was 0.41 h^{-1}. Thus, contrary to a common belief, thermophilic fungi do not, in general, grow faster than mesophilic fungi. The situation is similar to that in thermophilic bacteria (44, 240).

Respiration

Using the volume of carbon dioxide evolved over time as a measure of metabolism, Noack (188) compared a thermophilic fungus (*Thermoascus aurantiacus*) with a mesophilic fungus (*Penicillium glaucum*) grown in identical medium. He observed that the volume of carbon dioxide released by *P. glaucum* in 24 h was equivalent to 67% of its dry weight at 15°C and 133% at 25°C. He argued that if this fungus could grow at 45°C, the extrapolated value of carbon dioxide, according to van't Hoff rule, would be 532%. However, the actual value for *T. aurantiacus* at 45°C was 310%. From this, Noack inferred that at a given temperature the metabolism of a thermophilic fungus is actually slower than that of a mesophilic fungus. Subsequent studies have shown that the two types of fungi have nearly comparable respiratory rates at their respective temperature optima (203, 210).

Noack (188) observed that with increases in temperature, the increase in the respiration of *Thermoascus aurantiacus* was lower than that of mesophilic fungi. However, we found that the rates of oxygen uptake of homogeneous mycelial suspen-

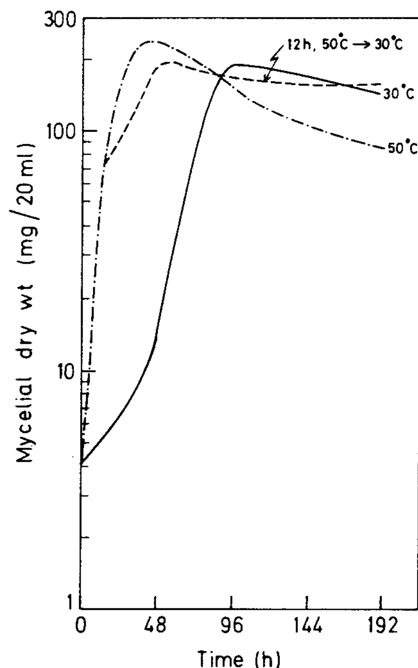

FIG. 1. Growth of *Thermomyces lanuginous* in submerged cultures at different thermal regimens (semilogarithmic plot). Reprinted from reference 208 with permission of the publisher.

sions of thermophilic fungi (*Thermomyces lanuginosus* and *Penicillium duponti*), measured by Warburg manometry, were markedly responsive to changes in temperature between their minimal (30°C) and optimal (50°C) temperatures of growth (203, 210). In contrast, the oxygen uptake rates of the mesophilic fungi tested (*Aspergillus niger*, *A. phoenicis*, and *Trichoderma viride*) were either independent of temperature changes between 15 and 40°C or affected to a lesser degree, at least during the period of measurement (Fig. 2). The biochemical basis of this difference is not known, but this behavior may be significant in relation to their growth in nature. If mycelia in the nutritionally poor environment of soil respond similarly, then fungi which maintain an optimal metabolic rate over a broad range of temperatures may be expected to have a competitive advantage over those which lack this ability. Mesophilic fungi, rather than thermophilic fungi, would be better adjusted to soil, where temperatures vary both spatially and temporally. Although a Warburg apparatus or an oxygraph is no longer standard equipment in laboratories, the investigations of respiratory metabolism still are very likely to lead to insights to physiological adaptations. In this context, another interesting observation was that whereas in the mesophilic fungus *Agaricus bisporus* both cytochrome and alternative respiratory pathways are present, in the thermophilic fungus *Scytalidium thermophilum* only the cytochrome-mediated respiratory pathway was present (74). Furthermore, high concentrations of CO and HCN severely inhibited the growth of *S. thermophilum* but not of *A. bisporus*.

Transport

Of the possible factors which restrict the growth of thermophilic fungi at ordinary temperatures, the reduction in the rate of uptake of nutrients appears to be particularly important. However, little information is available on the effect of growth temperature on the synthesis and activity of transport systems in fungi. Palanivelu et al. (196) identified a specific sucrose transport activity in *T. lanuginosus*, which was coinduced with invertase activity in mycelia exposed to β-fructofuranosides (sucrose or raffinose). Both activities appeared in sucrose-grown mycelia at about the same time, and both declined simultaneously following the exhaustion of sucrose in the medium. The uptake of sucrose was inhibited by ionophores that dissipate the proton gradient, suggesting that transport of sucrose is H^+ coupled. Furthermore, the uptake measured at 50°C followed Michaelis-Menten kinetics, with an apparent K_m of 250 μM (154). Transport of glucose in *T. lanuginosus* was a constitutive, specific, carrier-mediated process (K_m = 290 μM) that functioned as a proton-driven symport (A. K. Rajasekaran and R. Maheshwari, unpublished data). In preliminary experiments, an unexpected effect of the assay temperature on the rate of glucose uptake by *T. lanuginosus* was observed. Regardless of the growth temperature of the mycelia (50 or 30°C), glucose uptake by mycelia was saturated when assayed at 50°C. In contrast, it increased linearly with increasing concentrations of 2-deoxyglucose (tested up to 2 mM) at 30°C. The temperature-dependent sensitivity in the kinetics of glucose uptake may be due to a temperature-induced conformational change in glucose transporter.

The characteristics of sulfate permease in *Penicillium duponti* were similar to those in the mesophilic fungus *P. chrysogenum* (an $Na^+/H^+/SO_4^{2-}$ symport system derepressed by sulfur starvation, activated by divalent cations and inhibited by molybdate, vanadate, and tungstate, K_m of 57 μM) (263). They differed in that the permease of the thermophilic fungus was optimally active at 45°C and in its sensitivity to the ionic strength of the solution: mycelium that had been washed with deionized water lost transport activity.

Utilization of Carbon Sources

Thermophilic fungi develop in composts during the high-temperature phase, succeeding a mesophilic microflora (55,

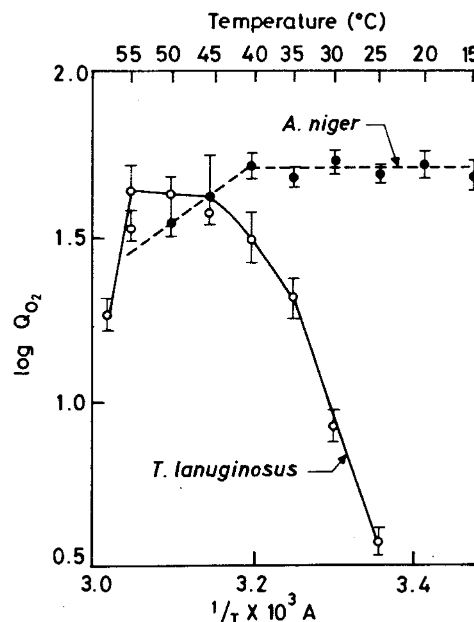

FIG. 2. Arrhenius plots of Q_{O_2} (microliters of oxygen taken up per mg [dry weight] of mycelium per hour) of shaker-grown mycelia of *Thermomyces lanuginosus* and *Aspergillus niger*. Reprinted from reference 203 with permission of the publisher.

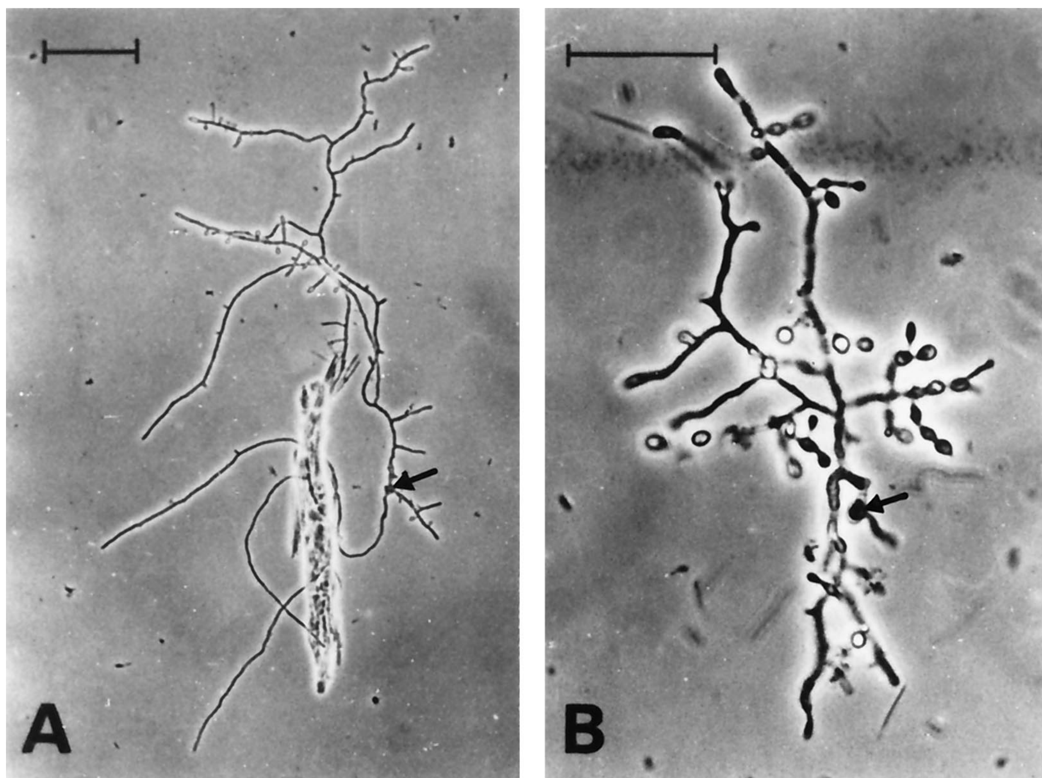

FIG. 3. Microcycle conidiation in *Sporotrichum thermophile*. The fungus was grown in shake cultures with shredded Whatman filter paper as the carbon source. (A) Phase-contrast micrograph of a 24-h-old germling that has produced oval asexual spores. The insoluble particle is a piece of cellulose fiber. (B) Phase-contrast micrograph showing precocious differentiation of asexual spores in a 72-h-old germling grown at 30°C. The germinated conidium is indicated by an arrow. Bars, 50 μm.

115). Since most of the initially available soluble carbon sources (sugars, amino acids, and organic acids) would have been depleted, the carbon source available for the growth of thermophilic fungi would be mainly the polysaccharide constituents of the biomass, of which cellulose is the chief constituent. Interestingly, some compost fungi are unable to utilize cellulose, for example, *Thermomyces lanuginosus* (55, 115, 204), *Talaromyces duponti*, *Malbranchea pulchella* var. *sulfurea*, *Mucor pusillus* (55), and *Melanocarpus albomyces* (156). The noncellulolytic species in compost can grow commensally by utilizing sugars released during the hydrolysis of hemicellulose and cellulose by the cellulolytic partner. For example, *T. lanuginosus* showed profuse growth in mixed cultures with a cellulolytic fungus, *Chaetomium thermophile* (115). Moreover, several noncellulolytic species readily utilize xylan, which is external to cellulose in the plant cell wall and is apparently a more accessible carbon source (199). Indeed, some fungi (*C. thermophile* and *Humicola insolens*) grow even better on xylan than on simple sugars (55). The secretion of thermostable extracellular polysaccharide-degrading enzymes and the simultaneous uptake of sugars would be important attributes of thermophilic fungi in self-heating masses of plant material. In *T. lanuginosus*, a single transporter was identified for glucose, xylose and mannose, the hydrolytic products of cellulose and hemicellulose (Rajasekaran and Maheshwari, unpublished).

Because the measurement of biomass of fungi growing on insoluble polysaccharides is indirect, it has rarely been done. We measured the growth of *Sporotrichum thermophile* on cellulose in terms of insoluble nitrogen or as an increase in mycelial dry weight after selectively estimating the amount of cellulose and subtracting its weight from that of the samples (32). Interestingly, the exponential growth rate of the fungus on cellulose (0.09 to 0.16 h^{-1}) was similar to that on glucose (0.1 h^{-1}), revealing the remarkable ability of this fungus to utilize cellulose as efficiently as glucose. The visual characteristics of the fungus were strikingly different in submerged cultures grown with cellobiose (repeating unit of cellulose) or cellulose (91). The mycelia in cellobiose-grown cultures retained a prolonged filamentous and healthy appearance, whereas in cellulose medium they rapidly autolysed and sporulated. Perhaps oligosaccharides derived from the hydrolysis of cellulose regulate the gene expression and metabolic process differently from when the fungus is growing on soluble sugars. Another interesting observation was the influence of culture temperature on fungal morphology when grown with cellulose. At a suboptimal temperature (30°C), the conidia of *S. thermophile* formed a very limited mycelium that precociously developed asexual reproductive structures (microcycle conidiation). Although the mechanism of this cellular response is not understood, microcycle conidiation (Fig. 3) may be a survival strategy of producing propagules in the shortest possible time under suboptimal conditions.

Mixed-Substrate Utilization

In composting plant material, the hydrolysis of polysaccharide constituents by the secreted enzymes would be expected to produce a mixture of sugars. We determined if thermophilic fungi utilize one sugar at a time or a mixture of sugars simultaneously (154). In the only study so far with fungi, a combination of glucose and sucrose was chosen, because the concentrations of these sugars in the medium can easily be determined

TABLE 3. Rate of protein degradation in some mesophilic and thermophilic fungi[a]

Fungus	Type of fungus	Temp (°C) of growth and assay of protein degradation	Degradation of protein (% h^{-1}) in:		
			Complete medium	Medium lacking N and S source	Buffer
Aspergillus niger	Mesophile	30	4.2	3.1	0.8
Trichoderma viride	Mesophile	30	8.6	8.5	5.7
Sporotrichum thermophile	Thermophile	50	3.3	4.3	1.3
Thermomyces lanuginosus	Thermophile	50	3.5	4.2	3.9

[a] Data from reference 208.

using commercially available enzymes. The fungi studied, *Thermomyces lanuginosus* and *Penicillium duponti*, concurrently utilized glucose and sucrose at 50°C, with sucrose being utilized faster than glucose. The phenomenon was studied further with *T. lanuginosus*. Its growth rates on single- or mixed-carbon sources were comparable. The rate of utilization of glucose and sucrose in the mixture was lowered unequally compared to when the sugars were provided singly, indicating that the two sugars reciprocally influenced their utilization in the mixture. The simultaneous utilization of sucrose in the presence of glucose occurred because (i) invertase was insensitive to catabolite repression by glucose and (ii) the activity of the glucose uptake system was repressed by glucose itself as well as by sucrose. Both sugars were also utilized concurrently at 30°C but at nearly identical rates. This observation indicates that the activity of nutrient transporters and the sensitivity of catabolic enzymes to glucose repression can be influenced differently at different temperatures.

Protein Breakdown

One of the early hypotheses put forward to explain thermophily in bacteria was that rapid breakdown of proteins at elevated growth temperatures is compensated by their fast resynthesis (9). This rapid-turnover hypothesis came to be known as the dynamic hypothesis of thermophily. To examine its applicability to thermophilic fungi, Miller et al. (176) compared protein breakdown rates in thermophilic (*Penicillium duponti*, *Malbranchea pulchella*, and *Mucor miehei*) and mesophilic (*Penicillium notatum* and *P. chrysogenum*) fungi by monitoring the breakdown of pulse-labeled protein. The growing cells in both types of fungi had a negligible rate of breakdown of bulk protein. In the nongrowing cells of both types, the breakdown rate was similar (5.2 to 6.7% per h). However, the breakdown rate of the soluble-protein fraction in thermophilic fungi was twice that in the mesophilic fungi. The authors suggested that the increased turnover rate of soluble protein is important in the survival of thermophilic fungi at the high temperatures.

The energy expended in increased protein turnover in thermophilic fungi would be expected to affect their growth yield compared to mesophilic fungi. However, the growth yields of thermophilic and mesophilic fungi examined were in a similar range (Table 2). Therefore, we reexamined protein breakdown in fungi (208) by selecting fungi which produced homogeneous mycelia, thereby obviating the sonication treatment used by Miller et al. (176) for rendering mycelia homogeneous for sampling and measuring radioactivity. Moreover, we attempted a general labeling of cellular proteins by adding radioactive amino acids at two different times and avoided the use of toxic concentrations of amino acids during the chase. Furthermore, we used the radioactivity of the whole cells as an index of the radioactivity of total protein rather than of the protein that was extractable by ultrasonic disruption of mycelia. The results pointed to a definite protein turnover in the growing cells of fungi, although the rate of protein turnover varied among the different species (Table 3). Under different conditions of incubation, the protein breakdown rate was somewhat lower in thermophilic fungi than in mesophilic fungi. Although we measured only protein breakdown, it is likely that the results would be similar had protein turnover been measured. As in bacteria (240), the rapid-turnover hypothesis in thermophilic fungi is not supported by experimental results. Nonetheless, specific enzymes may well have a high turnover rate.

Acquired Thermotolerance

Acquired thermotolerance refers to the enhanced survival of organisms at lethal temperature after a brief exposure to sublethal temperatures. A common phenomenon in mesophilic species is the synthesis of a set of proteins, called heat shock proteins (HSPs), following a sudden exposure of organisms to elevated temperature. The synthesis of HSPs is thought to be an adaptive response to increased thermotolerance and survival in the face of stressful conditions. Trent et al. (251) demonstrated that in common with thermophilic bacteria and archaea, thermophilic fungi also synthesize HSPs and acquire thermotolerance. They observed that conidia of *T. lanuginosus*, germinated at 50°C and heat shocked at 55°C for 60 min prior to exposure to 58°C, showed enhanced survival compared to non-heat-shocked conidia. Thermotolerance was eliminated if protein synthesis during the heat shock period was inhibited by cycloheximide. Pulse-labeling of proteins during the heat shock period, followed by their separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), showed increased synthesis of eight HSPs. Of these, three small HSPs ranging from 31 to 33 kDa dominated the heat shock response. Since *T. lanuginosus* is capable of growth up to 60 to 62°C, the role of HSPs induced at 55°C is not clear. A transient HSP synthesis was also observed in *Chaetomium thermophile* var. *thermophile* (190). In particular, a constitutive, abundantly expressed protein, HSP60, belonging to the intercompartmental transport proteins was thought to be important in thermophily.

In closing this section, we should mention that the impression gained is that only cursory explorations of the physiology of thermophilic fungi have been made, prompted primarily by a desire to explain their widespread distribution in soil—a habitat characterized by changing temperatures, uncertain supplies of nutrients and water, and the coexistence with a numerically high mesophilic microflora and fauna consisting of competitors and predators. Notwithstanding the reported latent abilities of some species to grow at ordinary temperatures, the controversy concerning the growth and reproduction of thermophilic fungi in soils is far from settled. Nonetheless, the attempts so far have dispelled certain early notions concerning

their rates of growth, their nutritional needs, and their overall metabolism.

SECRETORY ENZYMES

Enzymes of thermophilic fungi have been studied primarily to explore their suitability in bioprocesses and, to a lesser extent, to probe similarities and differences in physicochemical properties between enzymes from mesophilic and thermophilic fungi. Since culture filtrates can be obtained in substantial quantities, the enzymes that are secreted in the growth media have been studied more frequently than cell-associated enzymes, although such investigations have focused mainly on the identification of suitable thermophilic fungal sources for desired enzymes, the development of protocols for the purification of these enzymes, and the study of their general properties. In a few cases, however, the native or recombinant proteins have been examined to study interspecies differences in enzyme conformation, amino acid residues involved in substrate binding, packing of the hydrophobic core, electrostatic interactions, and stabilization of helices. In this review of thermophilic fungal enzymes, we have attempted to emphasize the realized as well as the potential contributions of such studies to biology and/or to biotechnology. In this section, the work on secretory enzymes, also referred to as extracellular enzymes, is summarized.

Protease

Proteases are variously classified on the basis of a critical amino acid required for the catalytic function (e.g., serine protease), the pH optimum of their activity (acidic, neutral, or alkaline protease), their site of cleavage (e.g., aminopeptidases, which act at the free N terminus of the polypeptide chain, or carboxypeptidases, which act at the C terminus of the polypeptide chain), or their requirement of a free thiol group (e.g., thiol proteinase).

Proteases have long been used in the food, dairy, and detergent industries and for leather processing. The need to overcome the limitation of obtaining chymosin, the milk-curdling enzyme from the stomach contents of milk-feeding calves, which is used in the industrial preparation of cheese, led to a search for substitutes. Arima et al. (17) screened about 800 microorganisms and obtained a soil isolate of *Mucor pusillus* that produced an enzyme with a high ratio of milk-clotting to proteolytic activity, enabling the production of high yields of curds. Subsequently, a strong milk-clotting activity was also observed in *M. miehei* (195). The milk-clotting activity of the enzyme was due to its selective attack on the *k*-casein fraction, which stabilizes the casein micelle in milk. The split *k*-casein loses its stabilizing activity, and the micelles of casein coagulate in the presence of calcium (233). The finding of a milk-clotting activity of utility resulted in serious efforts to isolate other thermophilic fungi in order to exploit them for industrially useful enzymes.

The Mucor rennins were produced by growing the fungus on wheat bran, from which they were extracted with water. The crude extract was then purified and crystallized (18, 20, 127, 195, 230, 234). Since the rennins hydrolyzed both casein and hemoglobin optimally at pH 3.7, they were classified as acid protease (EC 3.4.23.6). Both *M. pusillus* and *M. miehei* rennins hydrolyzed peptide bonds in synthetic peptides with an aromatic amino acid as the carboxyl donor (20, 195, 233). The *M. pusillus* enzyme was stable from pH 3.0 to 6.0 and showed maximum activity at 55°C (273). The enzyme was inhibited by the aspartic protease inhibitors diazoacetyl-DL-norleucine methyl ester and pepstatin (241). Sequence comparison with the other well-characterized aspartic proteases confirmed the presence of aspartic acid at their active site (25). *M. pusillus* and *M. miehei* rennins had similar molecular masses (38.5 and 42 kDa) and isoelectric points (3.9 and 4.1), respectively (84). Although the Mucor rennins (aspartic proteases) are structurally homologous, they differ from other fungal aspartic proteases and from mammalian proteases (25, 187, 249, 269). The specificity of Mucor rennins is similar to that of pepsin and calf rennin.

The Mucor rennins are being used as model systems in investigations of the heterologous expression of fungal protein, the mechanism of zymogen processing, the refolding of recombinant protein from inclusion bodies, the effects of glycosylation on secretion, and the activity and stability of mutant enzymes with amino acid insertions (6, 38, 100, 118). The Mucor rennin genes were cloned in *Escherichia coli* and sequenced (38, 100, 249). Their deduced amino acid sequence showed that the enzyme was synthesized as a zymogen that contained an N-terminal leader region constituting a typical signal peptide of 22 amino acids and a propeptide of 44 to 47 amino acids. This leader sequence was not present in the mature protein (361 amino acids). The expression of the Mucor protease gene in *E. coli* resulted in the accumulation of unsecreted, inactive polypeptide (38, 100), but when the gene was expressed in yeast, a form of the zymogen that was more glycosylated than the native enzyme was secreted at a concentration exceeding 150 mg/liter (267). The prosequence of the heterologous secreted protein was removed by autocatalytic processing at acidic pH, yielding an active rennin with properties almost identical to those of the native enzyme (118, 267). A mutation of two of the three glycosylated asparagine sites in the recombinant *M. pusillus* rennin significantly reduced the amount of secreted rennin by the yeast cells and decreased its milk-clotting activity and thermal stability compared to those of nonmutated *M. pusillus* rennin (6). It has been hypothesized that the flexible carbohydrate structures act as "heat reservoirs" and stabilize the conformation of rennins (269). The recombinantly produced *M. miehei* protease in *Aspergillus nidulans* was similar in specific activity to that produced by *M. miehei* (100). Subsequently, using an α-amylase promoter of *Aspergillus oryzae*, a recombinant *A. oryzae* strain was constructed that produced heterologous *M. miehei* protease in excess of 3 g/liter (61).

The high thermal stability of Mucor rennins turned out to be an undesirable property, since the residual enzyme activity, after cooking, can spoil the flavor of cheese during the long maturation process (268). Efforts are therefore being made to engineer Mucor rennins with lower thermostability but with the same milk-clotting potential. Nevertheless, the basic studies on Mucor enzymes have had interesting spinoffs. For example, the leader peptide of *M. pusillus* rennin was found to be useful for the secretion of a heterologous protein by yeast cells: when the human growth hormone gene was fused to the whole presequence and a part of the prosequence of *M. pusillus* rennin and expressed in yeast under the control of the yeast *GAL7* promoter, the level of the secreted hormone reached approximately 10 mg/liter (119).

A source of one of the most thermostable fungal acid proteases was a strain of *Penicillium duponti* isolated from compost (105, 106). The enzyme was produced in submerged cultures, containing rice bran, at 50°C with vigorous aeration and agitation. It was purified by alcohol precipitation, ion-exchange chromatography, and gel filtration (107). Subsequently, it was crystallized with an yield of 3.3 g from 200 g of crude protease preparation (79). The enzyme was most active at pH 2.5 on

casein and at pH 3.0 to 3.5 on hemoglobin. It had a molecular mass of 41 kDa and contained 4.3% carbohydrate. *P. duponti* protease retained its full activity after 1 h at 60°C and pH 4.5. By comparison, the acid protease of *M. pusillus* was irreversibly destroyed in 15 min at 65°C (230).

Two fungal sources of thermostable alkaline proteases were identified based on the zone of clearing of casein agar by culture filtrates as a semiquantitative assay of proteolytic activity: *Malbranchea pulchella* var. *sulfurea* and *Humicola lanuginosa* (193, 194). Both produced proteases during active growth in the presence of 2 and 8% (wt/vol) casein, respectively, suggesting that the enzyme was induced by external protein substrate (235). The production of protease by *M. pulchella* var. *sulfurea* was repressed by glucose, peptides, amino acids, or yeast extract (194). The *M. pulchella* protease, named thermomycolase, could be concentrated from the culture medium simply by vacuum evaporation at 45°C without a loss of activity. After dialysis and removal of pigments by adsorption on a cation-exchange column, a homogeneous preparation of the enzyme was obtained by affinity chromatography on a hydrophobic adsorbent with 78% yield. Its substrate specificity was investigated by analyzing the peptides formed in the reaction digests of glucagon and the A and B chains of oxidized insulin. At 45°C and pH 7.0, thermomycolase exhibited a general proteolytic activity rather than a well-defined specificity for any particular amino acid residue (235). The enzyme was classified as a serine protease based on inhibition of its activity by diisopropylfluorophosphate (DFP), which covalently attaches to a reactive serine residue. Thermomycolase was optimally active at pH 8.5 and was stable over a broad pH range (6.0 to 9.5 for 20 h at 30°C). As purified thermomycolase autolyzed, it resulted in low-molecular-mass peptides. The physicochemical characterization of the protein was therefore carried out using a DFP-inhibited enzyme. The molecular mass was 11 to 17 kDa when determined by gel filtration and 32 to 33 kDa when estimated from sedimentation equilibrium of DFP-thermomycolase and by SDS-PAGE (256). The thermostability of the enzyme depended on the concentration of calcium ions, a property shared by other thermostable enzymes, e.g., thermolysin, the neutral protease of *Bacillus thermoproteolyticus* (257). The $t_{1/2}$ (time required for the activity of the enzyme to fall to 50% of its original value at a given temperature) was 110 min at 73°C in the presence of 10 mM calcium. Calcium (10^{-4} M) was necessary to stabilize the enzyme against autolytic degradation at low temperatures: one calcium ion bound to the enzyme molecule with high affinity (258), markedly lowering the rate of autolytic degradation and of thermal or urea denaturation but causing no detectable change in the conformation of the enzyme. It was postulated that the bound calcium stabilized the enzyme by raising the local activation energy for unfolding.

An alkaline protease of *Humicola lanuginosa* (*Thermomyces lanuginosus*) was studied by two different groups, and their enzyme preparations differed in important properties. Shenolikar and Stevenson (226) purified the enzyme in one step based on its specific binding to an organomercury-Sepharose column, from which the enzyme was selectively eluted with a buffer containing mercuric chloride. The mercury-enzyme complex, reactivated by cysteine in the presence of EDTA, autolyzed rapidly. Based on the inhibition of the enzyme by some reagents, such as Hg^{2+} or *p*-chloromercuribenzoate, which react with free thiols, and its reactivation by cysteine in the presence of EDTA, the enzyme was identified as a unique thiol proteinase produced by a fungus (226). Both gel filtration and sedimentation analyses showed that the enzyme had a molecular mass of 237 kDa. It preferentially cleaved its substrate at the C-terminal end of the hydrophobic amino acid residues. However, Hasnain et al. (108) failed to find a protease that specifically bound to the affinity matrix used by Shenolikar and Stevenson (226). Rather, the protease activity was purified by hydrophobic affinity chromatography and shown to have a pH optimum of 8.0 and a molecular mass of 38 kDa and was inhibited by phenylmethylsulfonyl fluoride, an inhibitor that is specific to serine protease. Although the enzyme preparation obtained by Hasnain et al. was also inhibited by Hg^{2+} and *p*-chloromercuribenzoate, it was not inhibited by alkylating agents [iodoacetic acid, iodoacetamide or 5,5′-dithiobis(2-nitrobenzoic acid)], suggesting that it was a serine protease containing a partially buried cysteine. The authors (226) apparently favored the view that the fungus secreted only a single protease. Such differences in properties are generally explained on the basis of strain differences or culture-condition-induced modifications in enzyme structure. We shall encounter other examples of differences in enzyme properties from the same (?) fungus.

Lipase

Lipases (EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols and the synthesis of esters from glycerol and long-chain fatty acids. These enzymes exhibit the phenomenon of interfacial activation, i.e., the enhancement of catalytic activity on lipid aggregates (micelles) rather than on lipid monomers in aqueous solution. When used as ingredients of laundry detergents, lipases which are stable at pH 10 to 11, at temperatures from 30 to 60°C, and in the presence of surfactants are preferred.

Arima et al. (19) purified an extracellular lipase from *Humicola lanuginosa* strain Y-38, isolated from compost in Japan. The enzyme was produced in a medium containing soybean oil, starch, corn steep liquor, and antifoaming agent. It was purified to homogeneity from 80-h-old culture medium by successive steps of ammonium sulfate precipitation, dialysis, ion-exchange chromatography, and gel filtration chromatography, with 30% recovery. The protein, a single polypeptide (molecular weight, 27,500), was optimally active at pH 8.0 and was stable in the pH range of 4 to 11. Its temperature optimum for activity was at 60°C. It showed appreciable activity at up to 65°C but was inactivated on heating at 80°C for 20 min (149). The enzyme could be stored frozen for more than 6 months. The enzyme molecule contained disulfide linkages but no free —SH group.

Omar et al. (191, 192) reported that the productivity and thermostability of lipase differed with different strains of *H. lanuginosa*. These workers developed an optimized medium containing sorbitol, corn steep liquor, silicone oil as an antifoaming agent, and whale or castor oil as enzyme inducer. With the pH maintained between 7 and 8 and the temperature set at 45°C, maximum enzyme production by their strain occurred after 30 h. Following acetone precipitation and successive chromatographic steps, they obtained a more thermostable enzyme (stable at 60°C for 20 h) than was obtained by Arima et al. (19). Their preparation was optimally active at pH 7.0. The enzyme showed increased activity in organic solvent-aqueous reaction systems, but hydrolysis in complete organic phase reactions did not occur.

A lipase gene from *H. lanuginosa* was cloned and expressed in *Aspergillus oryzae* (122). The lipase expressed in the heterologous host was purified by a two-step procedure involving hydrophobic interaction chromatography and ion-exchange chromatography (198). The structure of *H. lanuginosa* lipase (73) was similar to that of *R. miehei* lipase. Structural studies have been directed toward an understanding of the phenom-

enon of interfacial activation. Modification in the lid (described below) of *H. lanuginosa* lipase by site-directed mutagenesis provided direct evidence of the importance of a number of residues within the lid in terms of substrate binding and specificity (120). By spectroscopy and molecular dynamics simulation, Peters et al. (198) showed that a single mutation of a serine residue in the active site leads to substantial alterations in the motion of the lid and binding affinity of enzyme.

Rhizomucor miehei, formerly called *Mucor miehei*, also produces active extracellular lipase. The isolation and purification methods for *M. miehei* lipase have been described by Huge-Jensen et al. (123). The lipase produced predominantly in form A (see below) was purified by anion-exchange chromatography followed by affinity chromatography and further purified by hydrophobic interaction chromatography (39). If the purification steps were carried out at pH 4.5 instead of pH 7.0, form A was partially deglycosylated and converted to form B. The two forms showed a high degree of antigenic similarity and were optimally active at pH 7.0. However, form A, in contrast to form B, required a prior alkaline (pH 10.5) treatment for maximal activation. The apparent molecular mass of both lipases was ~32 kDa. The carbohydrate contents of purified *R. miehei* lipase forms A and B were 11% and 4% (wt/wt), respectively. The lipases rapidly hydrolyzed a broad spectrum of lipids found in animal fat and vegetable oil. The enzyme remained active even after exhaustive drying (254).

Boel et al. (39) constructed an *R. miehei* cDNA library in *E. coli*. From the DNA sequence data and the deduced amino acid sequence, they inferred that, unlike the characterized bacterial and mammalian enzymes, the *R. miehei* lipase was synthesized as a zymogen with a signal peptide of 24 amino acid residues and a further 70-amino-acid propeptide. Maturation involved a proteolytic cleavage to remove the propeptide. In further work (122), a recombinant plasmid containing the cDNA of *R. miehei* lipase was expressed in the filamentous fungus *Aspergillus oryzae* and the enzyme was obtained in large quantities. Heterologous expression did not affect the characteristics of the enzyme, showing that the precursor was correctly processed in *A. oryzae*.

R. miehei lipase has 269 amino acid residues. It was the first lipase whose three-dimensional structure was deduced by X-ray analysis (42). The lipase is an α/β-type protein, having a core of central, mostly parallel β-sheets connected by a variety of hairpins, loops, and helical segments. Although the overall protein structure is quite unrelated to that of the serine proteases, the lipase catalytic center has the same three amino acids (serine, histidine, and aspartic acid) which characterize serine proteases as well. The finding of this "catalytic triad," which set up an H^+ shuttle or the charge relay system at the active site of lipase, as in the enzymes of the trypsin family, suggests their convergent evolution. An interesting feature of the protein molecule is that the catalytic site is covered by a short α-helical loop that acts as a "lid." When the enzyme is adsorbed at the oil-water interface, the lid moves, allowing access of the substrate to the active site and at the same time exposing a large hydrophobic surface which apparently facilitates the binding of the lipase to the lipid interface (72).

H. lanuginosa lipase (Lipolase; Novo Industri A/S) is being used in detergent formulations in conjunction with other microbial enzymes (e.g., protease, amylase, and cellulase). In addition, lipases have applications in the food industry and are used for the biocatalysis of stereoselective transformations (128). Lipase (Lipozyme; Novo Industri A/S) from *R. miehei* is used to produce a cocoa butter substitute from a cheaper edible oil, in which oleate exclusively occupies the *sn*-2 position but palmitate rather than stearate predominates at the *sn*-1 and *sn*-3 positions (104, 128). Efforts in protein engineering of lipases are being made to obtain improved binding to negative charges on the lipid surface, to open the lid and activate the enzyme, and to increase stability to high pH and to anionic and nonionic surfactants.

α-Amylase

α-Amylase (EC 3.2.1.1) hydrolyzes α-1,4-glycosidic linkages in starch to produce maltose and oligosaccharides of various lengths. All species of thermophilic fungi studied so far secrete amylase (3, 4, 23, 46, 85, 129, 220). However, only *T. lanuginosus* α-amylase has been characterized. The addition of Tween 80 to agitated submerged cultures increased α-amylase production 2.7-fold (22). Although a multiplicity of α-amylases is common in fungi, only one electrophoretically similar form of the enzyme was detected in culture filtrates of seven strains of *T. lanuginosus* (177). α-Amylases from two strains have been purified, but the estimations of their molecular masses gave ambiguous results. While the enzyme purified from stationary cultures of strain 1457 had a molecular mass of 54 to 57 kDa by SDS-PAGE (130), the enzyme purified from shaker-grown cultures of strain IISc 91 gave different values by different methods: ~24 kDa by SDS-PAGE, ~72 kDa by gel filtration, and ~42 kDa by Ferguson analysis on native PAGE (177). Since the partial amino acid sequence of IISc 91 α-amylase showed a single N-terminal amino acid, it was suggested that the native enzyme is a homodimer with an apparent molecular mass of ~42 kDa. This was the first report of a dimeric form of α-amylase in fungi. The α-amylases were stabilized by calcium. For example, 10 mM calcium increased the thermostability of IISc 91 α-amylase by eightfold. After addition of Ca^{2+}, its half-life at 65°C was 4.5 h. A novel observation was that IISc 91 α-amylase underwent structural changes upon heating to 94°C: the native, dimeric enzyme was progressively and irreversibly inactivated, and the subunits dissociated and reassociated to produce an inactive, 72-kDa trimeric species which fragmented on further heating. The enzyme produced exceptionally high levels of maltose from raw potato starch (177).

Glucoamylase

Glucoamylase (E.C. 3.2.1.3) is an exo-acting enzyme which hydrolyzes α-1,4-glycosidic linkages and, less frequently, α-1,6-glycosidic linkages from the nonreducing end of starch, producing β-D-glucose as the sole product. During growth in a medium containing starch, *T. lanuginosus* also produced glucoamylase (213, 214, 246), which was separated from α-amylase by conventional procedures of protein purification. Although maltose had been reported to be a better inducer of glucoamylase in this fungus (103), growth in a starch medium allowed both glucoamylase and α-amylase to be produced simultaneously. Both enzymes were obtained in milligram quantities from 2 liters of culture filtrates (177). Although glucoamylases of *T. lanuginosus* had similar carbohydrate contents (10 to 12%), different authors have reported different molecular masses for the enzyme: ~57 kDa by gel filtration and SDS-PAGE (217), 70 to 77 kDa by SDS-PAGE (131), ~45 kDa by SDS-PAGE (178), and 72 kDa by SDS-PAGE (76). Except for this difference in molecular mass, the thermostabilities of the glucoamylases were similar. For example, IISc 91 glucoamylase was stable for ~7 h at 60°C (177). Unlike α-amylase of *T. lanuginosus*, glucoamylase of the same organism was less stable in the presence of added calcium.

Although *T. lanuginosus* produced both α-amylase and glucoamylase activities simultaneously, the enzymes did not ex-

hibit the synergism observed in the mesophilic fungus *Aspergillus* sp. (1). The thermal resistance of *T. lanuginosus* glucoamylase was increased severalfold by its entrapment in polyacrylamide gels (215). Glucoamylase effected up to 76% conversion of soluble or raw potato starch to glucose in 24 h, indicating that it was insensitive to end product inhibition (177). The properties of the enzyme suggested its usefulness in the commercial production of glucose syrups.

Another thermophilic fungus with a high potential in starch saccharification is *Humicola grisea* var. *thermoidea*. A strain of this fungus, isolated from soil from Brazil, produced 2.5- to 3.0-fold-higher glucoamylase activity when grown in a rich medium containing maltose as the principal carbon source than when grown on starch (250). The major starch-hydrolyzing enzyme had a molecular mass of 63 kDa, with pH and temperature optima of 5.0 and 55°C, respectively. The efficiency (V_{max}/K_m) of purified protein to hydrolyze starch was twice that of maltose. Kinetic experiments suggested that both starch and maltose were hydrolyzed at the same catalytic site. Another strain of *H. grisea* var. *thermoidea* produced a glucoamylase (74 kDa) that was remarkably insensitive to end product inhibition; it retained 65% activity in the presence of 950 mM glucose (47). Moreover, the enzyme was maximally active at pH 6.0 and 60°C. Increasing the copy number of the encoding gene through transformation (11) increased glucoamylase production by this fungus nearly threefold.

Cellulase

The cellulase system in fungi is considered to comprise three hydrolytic enzymes: (i) the endo-(1,4)-β-D-glucanase (synonyms: endoglucanase, endocellulase, carboxymethyl cellulase [EC 3.2.1.4]), which cleaves β-linkages at random, commonly in the amorphous parts of cellulose; (ii) the exo-(1,4)-β-D-glucanase (synonyms: cellobiohydrolase, exocellulase, microcrystalline cellulase, Avicelase [EC 3.2.1.91]), which releases cellobiose from either the nonreducing or the reducing end, generally from the crystalline parts of cellulose; and (iii) the β-glucosidase (synonym: cellobiase [EC 3.2.1.21]), which releases glucose from cellobiose and short-chain cellooligosaccharides (33). Although β-glucosidase has no direct action on cellulose, it is regarded as a component of cellulase system because it stimulates cellulose hydrolysis (see below).

Based on the view prevalent in the 1970s that the levels of extracellularly produced cellulase enzymes determine the extent of solubilization of cellulose, several fungi were isolated and screened for high total cellulase activity in an attempt to develop a practical process for the enzymatic conversion of cellulose into glucose (159, 160). Mandels (159) observed that some species of thermophilic fungi degraded cellulose rapidly but that their culture filtrates had low cellulase activity. This was contradicted by reports that the thermophilic fungi *Sporotrichum thermophile* (67) and *Talaromyces emersonii* (88) produced cellulase activity nearly comparable to that of the mesophilic fungus *Trichoderma reesei*, regarded as the best source of fungal cellulase. Although cellulase productivity varies among strains (189), using uniform procedures for the measurement of cellulase activity, Bhat and Maheshwari (32) demonstrated that the endoglucanase and exoglucanase activities in the culture filtrate of their best strain of *S. thermophile* were about 10-fold lower and the β-glucosidase activity was about 1.6-fold lower than in *T. reesei*. Despite these lower activities, *S. thermophile* degraded cellulose faster and grew at five times the rate of *T. reesei*. That *S. thermophile* has a powerful cellulolytic system was corroborated by the observation that its growth rates on insoluble cellulose and glucose were similar. Of greater significance, these observations raised strong doubts about the notion that secreted levels of cellulase determine the rate or extent of cellulolysis.

A question that had remained unresolved was whether cellulase formation in fungi is directly correlated with mycelial growth; i.e., were the enzymes produced during the trophophase or the idiophase? To determine this, the growth of *S. thermophile* on cellulose was arrested at different times by cycloheximide addition (32). When fungal growth was curtailed, some cellulose remained insolubilized in the medium, although cellulase that had already been secreted prior to growth arrest was present in the culture medium. It was inferred that degradation of cellulose is intimately associated with fungal growth (32).

In general, crystalline cellulose was found to be a superior carbon source for induction of cellulase enzymes in thermophilic fungi than were its amorphous or "impure" forms (90, 93, 166, 167, 211, 217). The exceptions are *Thermoascus aurantiacus* (137, 139, 140), *Humicola insolens* (113), and *H. grisea* var. *thermoidea* (270), which produced high cellulase and xylanase activities even on hemicellulosic substrates without cellulose. In some strains of *S. thermophile*, the disaccharides cellobiose and lactose also induced cellulase, although less efficiently (50, 189). The time course of the appearance of various cellulase components varied in different species: thus, in *H. insolens* (271) and *T. aurantiacus* (138), all three cellulase components appeared simultaneously, while in *Chaetomium thermophile* var. *coprophile*, β-glucosidase activity preceded endo- and exoglucanase activities (92) and in *S. thermophile* it lagged behind endoglucanase and exoglucanase, which were typically formed during active growth. Rather, the appearance of β-glucosidase in medium coincided with the time of extensive autolysis of mycelium (32, 91). For purification of cellulases, aged cultures have been used in which cellulose was nearly completely solubilized and the β-glucosidase activity was maximal. The cellulase components have been separated using combinations of ion-exchange chromatography and gel filtration chromatography and/or preparative gel electrophoresis, but the protein yields have generally been low. Among the exceptions was a soil isolate of *T. aurantiacus* from India, which was grown in shake flasks containing shredded paper and peptone. From about 2 liters of culture filtrate, 1,622 mg of desalted crude protein was obtained, which, on further processing, yielded 30 mg of β-glucosidase, 335 mg of exoglucanase, 161 mg of endoglucanase, and 258 mg of crystalline xylanase (139). Yoshioka and Hayashida (271) purified cellulases from a strain of *H. insolens* grown on wheat bran. From 2.5 g of culture extract protein, 9, 11, and 18 mg, respectively, of pure endoglucanase, exoglucanase, and β-glucosidase were obtained (110, 113, 271).

Like the mesophilic fungi, the thermophilic fungi produce multiple forms of the cellulase components. However, two different strains of *T. aurantiacus* produced one form each of endoglucanase, exoglucanase, and β-glucosidase, but the forms from the two strains had somewhat different properties (139, 248). The multiplicity of individual cellulases might be a result of posttranslational and/or postsecretion modifications of a gene product or might be due to multiple genes. For example, *T. emersonii* produced multiple endoglucanases, exoglucanases, and β-glucosidases (65, 66, 165–168). Its culture filtrate protein was resolved by ion-exchange chromatography into four endoglucanases (Table 4) which, unlike their variable carbohydrate contents (28 to 51%), had similar molecular masses (68 kDa by gel filtration and 35 kDa by SDS-PAGE), isoelectric points, pH and temperature optima, thermal stabilities, and specific activities (178). The different endoglucanases were thought to re-

TABLE 4. Salient properties of β-1,4 endoglucanases of thermophilic fungi

Fungus	Optimal pH	pI	Optimal temp (°C)	$t_{1/2}$ (min)	Mol mass (kDa)	% (wt/wt) Carbohydrate	Reference(s)
Chaetomium thermophile							
var. *dissitum*	5.0 ?	4.55	55	NR[a]	41	NR	81
var. *coprophile*	6.0	NR	60	18 (60°C)	36	NR	94
Humicola grisea var. *thermoidea* (mutant)	5.0 ?	NR	NR	Stable for 10 min (70°C)	63	NR	109
Humicola insolens							
YH-8 (Japanese isolate)	5.0	NR	50	5 (95°C)	57	39.0	112, 113
Indian isolate	5.6 ?	NR	50	NR	45	16	211
Myceliophthora thermophila	4.8	NR	65	Stable for 60 min (70°C)	100	NR	219
Talaromyces emersonii							178
EG I	5.5–5.8	3.19	75–80	104 (75°C)	35	27.7	
EG II	5.5–5.8	3.08	75–80	93 (75°C)	35	29.0	
EG III	5.5–5.8	2.93	75–80	75 (75°C)	35	44.7	
EG IV	5.5–5.8	2.86	75–80	66 (75°C)	35	50.8	
Thermoascus aurantiacus							
New Zealand isolate	4.5	NR	65	Stable for 60 min (65°C)	34	1.8	228, 248
Indian isolate	2.9	NR	76	32 (80°C)	32	1.7	139

[a] NR, not reported.

flect differential posttranslational or postsecretion modifications of a single gene product. However, cloning and expression of seven endoglucanase genes of *H. insolens* demonstrated a genetic basis of multiple forms. The seven cloned endoglucanases produced in *Aspergillus oryzae* were purified from culture medium by affinity chromatography on cellulose (70, 223, 224) and were found to differ in cleavage site specificity and kinetic constants (k_{cat}/K_m).

The endoglucanases (30 to 100 kDa) of thermophilic fungi are thermostable, with optimal activity between 55 and 80°C at pH 5.0 to 5.5 and with carbohydrate contents from 2 to 50% (Table 4). The exoglucanases (40 to 70 kDa) are optimally active at 50 to 75°C and are thermostable; these enzymes are glycoproteins (Table 5). The partially deglycosylated endo- and exoglucanases of *H. insolens* showed significantly lower thermal and pH stabilities, suggesting a role of carbohydrate in stabilization of these proteins (112). The molecular characteristics of β-glucosidases are more variable (Table 6), with molecular masses ranging from 45 to 250 kDa and carbohydrate contents ranging from 9 to 50%. An isolate of *H. insolens* produced an unusual β-glucosidase (88 kDa) that was stimulated by β-mercaptoethanol and was resistant to SDS even in the presence of β-mercaptoethanol (212). Although inactivated by 8 M urea, the enzyme regained full activity after dilution. A β-glucosidase of rather broad specificity was isolated from *H. grisea* var. *thermoidea* (197). Its affinity (V_{max}/K_m) for glycosyl residues in aryl glycosides was in the order β-D-fucosyl > β-D-glucosyl > β-D-galactosyl. The rates of hydrolysis of substrate mixtures were not additive, suggesting that the enzyme had a common catalytic site for the substrates assayed. The enzyme also hydrolyzed cellobiose and lactose, had 23% carbohydrate, and had a molecular mass of 55 kDa. In general, except for the thermostability, the molecular characteristics of cellulase components of thermophilic and mesophilic fungi are similar.

A remarkable feature of the cellulase system is synergism; i.e., the action of a mixture of two or more cellulases is greater than the sum of the action of each component. Eriksen and Goksøyr (80, 81) purified one form each of endoglucanase, exoglucanase, and β-glucosidase from *Chaetomium thermophile* var. *dissitum*. Each of the cellulase components, when used singly, showed little activity on cotton, but when all the components were combined, extensive degradation of cotton occurred. Yoshioka et al. (270) purified an endoglucanase from *H. grisea* var. *thermoidea* which, unlike other fungal endoglucanases, was completely absorbed onto crystalline cellu-

TABLE 5. Salient properties of β-1,4-exoglucanases from thermophilic cellulolytic fungi

Fungus	pH_{Opt}, pH stability, pI	T_{Opt}, stable temp (time)	Mol mass (kDa)	% Carbohydrate (wt/wt)	Reference(s)
Chaetomium thermophile					
I	pH_{Opt} = 5.8	T_{Opt} = 75°C, stable at 60°C (1 h)	60	17.0	93
II	pH_{Opt} = 6.4	T_{Opt} = 70°C, stable at 60°C (1 h)	40	23.0	
Humicola insolens	pH_{Opt} = 5.0, stable pH = 3.5–9.5	T_{Opt} = 50°C, stable at 65°C (5 min)	72	26.0	110
Sporotrichum thermophile	pH_{Opt} = 4.0–5.0, pI = 4.5	T_{Opt} = 63°C, stable at 63°C (3 h)	64	8.0	90
Thermoascus aurantiacus					
New Zealand isolate	pH_{Opt} = 5.0, stable pH = 5–12	T_{Opt} = 60°C, stable at 60°C (1 h)	49	2.6	228, 248
Indian isolate	pH_{Opt} = 4.1	T_{Opt} = 65°C, stable at 50°C (8 h)	58	5.2	139

TABLE 6. Salient properties of extracellular β-glucosidases from thermophilic fungi

Fungus	Specificity	pH_{Opt}, pI	T_{Opt}, $t_{1/2}$, stable temp (time)	Mol mass (kDa)	% Carbohydrate (wt/wt)	Reference(s)
Humicola grisea var. *thermoidea*	PNPG[a]	pH_{Opt} = 4.0–4.5	T_{Opt} = 60°C, $t_{1/2}$ = 15 min at 65°C	156	NR[b]	86
Humicola insolens						
Japanese isolate	Cellobiose, β-glucosides; no action on cellulose	pH_{Opt} = 5.0, pI = 4.2	T_{Opt} = 50°C, stable at 60°C (5 min)	250	2.5	110
Indian isolate	Cellobiose, β-glucosides, Cellulose, xylan	pH_{Opt} = 5.6, pI = 8.1	T_{Opt} = 50°C	45	10.0	211
Humicola lanuginosa	Cellobiose (K_m = 0.44 mM), PNPG (K_m = 0.5 mM), xylan, β-D-xyloside	pH_{Opt} = 4.5	T_{Opt} = 60°C	110	9.0	14
Sporotrichum thermophile	Cellobiose (K_m = 0.83 mM), PNPG (K_m = 0.3 mM), cellodextrins; inactive on cellulose	pH_{Opt} = 5.4	T_{Opt} = 65°C	240		33
Talaromuces emersonii						65
(I) Extracellular	Cellobiose	pH_{Opt} = 4.1	T_{Opt} = 70°C	135	50.0	
(II) Extracellular				100		
(III) Extracellular	Cellodextrins	pH_{Opt} = 5.1	T_{Opt} = 70°C	45	26.0	
(IV) Intracellular	Cellobiose	pH_{Opt} = 5.7	T_{Opt} = 35°C	57	12.0	
Thermoascus aurantiacus						
New Zealand isolate	PNPG (K_m = 0.5 mM), cellobiose, xylan, lichenan	pH_{Opt} = 5.0	T_{Opt} = 70°C	87	33.0	228, 248
Indian isolate	Cellobiose > PNPG	pH_{Opt} = 4.2	T_{Opt} = 71°C	98	15.1	139

[a] PNPG, *p*-nitrophenyl-β-D-glucopyranoside.
[b] NR, not reported.

lose but had no activity on this material. However, when this endoglucanase was incubated with an exoglucanase, even of a different origin, it showed a high synergistic action on crystalline cellulose. On the other hand, cellulase components of *T. aurantiacus* showed no synergism (228). Why some cellulase enzymes show synergism whereas others do not is of great interest. In this regard, the observations of Hayashida and Mo (109) on mutants of *H. grisea* var. *thermoidea* are relevant. One mutant produced an endoglucanase (128 kDa) that adsorbed tightly onto crystalline cellulose, disintegrated fibrils (mechanical dispersion), and showed strong synergistic activity with the *T. reesei* cellulase. Another mutant produced an endoglucanase (63 kDa) that did not adsorb onto or disintegrate cellulose and showed less synergism. The observations suggested that a crucial aspect of synergism is adsorption of cellulase components onto cellulose. Endoglucanase, exoglucanase, and β-glucosidase may form a ternary complex with the substrate (178).

During the 1970s to 1980s, practical considerations of enzymatic saccharification of cellulose by cellulase preparations from *T. reesei* generated much interest in β-glucosidase because the addition of this enzyme to reaction mixtures stimulated the rate and extent of cellulose hydrolysis (232). The potentiating effect of β-glucosidase was explained on the basis that it cleaved cellobiose and relieved end product inhibition of cellulase activity. However, in *S. thermophile*, β-glucosidase stimulated cellulose hydrolysis by some endo- and exoglucanases even when little or no cellobiose accumulated in the reaction mixture (31). It was hypothesized that β-glucosidase physically associated with endo- and exoglucanases to form a catalytically more active cellulase complex. The presence of multienzyme complexes of cellulase components anchored on the hyphal wall, similar to the cellulosome in bacteria (34, 145), may explain the powerful cellulolysis by some thermophilic fungi.

The endoglucanases of *H. insolens* were cloned and expressed in *Aspergillus oryzae*. The enzyme had two domains (69): a catalytic domain and a cellulose-binding domain joined by a 33-amino-acid linker sequence (40). Endoglucanase 1 (EG1) was a single-domain enzyme composed of two antiparallel β-sheets (152). Although the structures of *H. insolens* and *T. reesei* endoglucanases (EG1) were very similar (143), their pH optima were different, at 7.5 and 4.5, respectively (225). This difference was ascribed to a subtle change, i.e., an active-site alanine of *T. reesei* enzyme replaced by histidine in *H. insolens*. Based on analysis of the enzyme structure by X-ray diffraction, it was proposed that this histidine could furnish protons to a glutamate residue at the active site and shift the pH optimum to the alkaline region. In both endoglucanases, the active site was located in a long open groove. The structure of *H. insolens* EGV with an oligosaccharide bound to it suggested that the enzyme would favor the binding of a transition state substrate with an elongated glycosidic bond (69). The knowledge of the structures of cellulases and the use of recombinant DNA technology may allow the design of cellulases for use in detergent formulations; these might include cellulases with higher pH optima (143). (In combination with detergent, cellulase removes unwanted "pills" that are formed in clothes due to repeated washing and wearing and which collect dirt.)

Cellobiose Dehydrogenase

The observation that cellulolysis by culture filtrates of the white rot fungus *Sporotrichum pulverulentum* was enhanced in

the presence of oxygen led to the discovery of an oxidative enzyme(s) that oxidized cellobiose and cellodextrins using quinones as the electron acceptor (cellobiose + acceptor → cellobionolactone + reduced acceptor) (82). Subsequently, cellobiose dehydrogenases (CDHs) (EC 1.1.99.18) were isolated from *S. thermophile* (48, 49, 64, 222) and *H. insolens* (222) and shown to be hemoflavoproteins of 92 to 95 kDa. The CDHs had temperature optima of 60 to 65°C (222, 238). The pH optima of *H. insolens* CDH was 7.5 to 8.0, and that of *M.* (*S.*) *thermophile* was ~4.0 (125, 222). Cloning and sequencing of *S. thermophile* CDH (238) showed that the enzyme has three domains: an N-terminal flavin domain, which contains the catalytic site (125); a middle heme domain; and a C-terminal cellulose-binding domain with homology to the cellulose-binding domain of cellulase of *T. reesei*. The role of CDH in cellulolysis by fungi is not clear. Although the *H. insolens* CDH could use oxygen as an electron acceptor (222), the identity of the reduced oxygen species is not known. It is thought that oxygen is reduced to hydrogen peroxide, which in the presence of Fe^{3+} can generate hydroxyl radicals ($H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + HO^- + Fe^{3+}$) that, in cooperation with cellulase, can depolymerize cellulose (124, 147, 161).

Xylanase

Next to cellulose, xylan is the most abundant structural polysaccharide in nature. Its complete degradation requires the cooperative action of a variety of hydrolytic enzymes: the endoxylanases (EC 3.2.1.8), which randomly cleave β-1,4-linked xylose (the xylan backbone); the β-xylosidases (EC 3.2.1.37), which hydrolyze xylooligomers; and the different side-branch-splitting enzymes, e.g., α-glucuronidase and α-arabinosidase, acetylxylan esterase, and acetyl esterase, which liberate other sugars (glucuronic acid arabinose) that are attached as branches to the backbone (35). Xylanases of thermophilic fungi are receiving considerable attention because of their application in biobleaching of pulp in the paper industry, wherein the enzymatic removal of xylan from lignin-carbohydrate complexes facilitates the leaching of lignin from the fiber cell wall, obviating the need for chlorine for pulp bleaching in the brightening process. They also have applications in the pretreatment of animal feed to improve its digestibility.

A variety of materials have been used for induction of xylanases: pure xylan (98, 142, 163, 164, 205) and xylan-rich natural substrates, such as sawdust (274), corn cob (26, 99, 205), wheat bran (272), sugar beet pulp (253), and sugarcane bagasse (57, 199). Paper of inferior quality was an excellent carbon source and inducer for xylanase in *Thermoascus aurantiacus* (139), *Humicola lanuginosa* (13, 15), and *Paecilomyces varioti* (144). In *Melanocarpus albomyces* (156) and *Thermomyces lanuginosus* (205), xylose, the pentosan unit of xylan, could also induce xylanase. Xylanases are often coinduced with cellulases by pure cellulose, as in *T. aurantiacus* (248), *Chaetomium thermophile* var. *coprophile* (95), and *H. insolens* (183). In *M. albomyces* (156) and *T. lanuginosus* (98, 99, 206), xylanase, but little or no cellulase, was produced. Crude culture filtrates of these fungi can therefore be used for biobleaching of paper pulp. The majority of xylan-degrading enzymes from thermophilic fungi are endoxylanases.

Malbranchea pulchella var. *sulfurea* also produced an extracellular xylosidase (162–164), but in *H. grisea* var. *thermoidea* (12, 179) and *Talaromyces emersonii* (253) the xylosidase was periplasmic. Interestingly, the best xylanase-producing strains of *T. lanuginosus* secreted small amounts of xylan-debranching enzymes and did not produce β-mannan- and arabinan-degrading enzymes, whereas the low-xylanase-producing strains exhibited a higher degree of xylan utilization and also the ability to produce a mannan-degrading enzyme system (204). This suggests that utilization of xylan is facilitated by the removal of other polysaccharides that are tightly bound or cross-linked to xylan.

Outstanding yields of xylanases, requiring only three- to fourfold purification of culture filtrate protein, have been obtained from some wild isolates of thermophilic fungi (139, 144, 242). In *T. aurantiacus*, 258 mg of crystalline xylanase was obtained from 1,622 mg of desalted crude culture filtrate protein, with 75% yield (139). In *P. varioti*, the secreted protein was mostly xylanase with small amounts of β-glucosidase (144). When the culture filtrate after ammonium sulfate precipitation (50 to 70 mg of protein/ml) was kept at 4°C for 10 to 12 h, xylanase crystallized out of the solution without the need for prior chromatographic purification procedures. Strains of *H. lanuginosa*, *P. varioti*, and *T. aurantiacus* have yielded 2, 52, and 136 mg of pure xylanase from 1 liter of culture filtrates (183). All three xylanases were crystallized.

As in the mesophilic fungi, a multiplicity of xylanases has also been observed in some thermophilic fungi (Table 7). Multiple forms of xylanases differ in stability (199), catalytic efficiency (179, 199), and absorption onto and activity on substrates (77, 253). A possible role for the production of xylanase isozymes of different molecular sizes (199) might be to allow their diffusion into the plant cell walls of highly variable structures. The majority of xylanases have pH optima ranging from 4.5 to 6.5. *T. emersonii* xylanases are unusual in having acidic pH optima. The temperature optima of most xylanases range from 55 to 65°C. Xylanases of some strains of *T. aurantiacus* and *T. lanuginosus* are optimally active at 70 to 80°C. The molecular masses of xylanases cover a wide range, from 21 to 78 kDa (Table 7). With the exception of the dimeric Xyl I and Xyl II of *T. emersonii*, most xylanases are single polypeptides. Xylanase I of *C. thermophile* var. *coprophile* and (95) xylanase II of *H. insolens* were remarkable in their low molecular mass (7 kDa) (77). Xylanases are glycoproteins. The carbohydrate content of the three xylanases of *Talaromyces byssochlamydoides* varied from 14 to 37%. Two endoxylanases of *T. emersonii* were unusual in that they had no action on xylan unless the arabinose substituents were removed and also in their ability to hydrolyze aryl β-D-xylosides. Xylanases have not shown cooperative interaction in the hydrolysis of xylan (96, 97, 199). Polyclonal antibodies showed antigenic cross-reactivity among xylanases of *T. aurantiacus*, *P. varioti*, *H. insolens*, and *H. lanuginosa* (183).

Generally proteins have a long shelf-life in the dry state. However, the lyophilized xylanase of *H. lanuginosa* was inactivated after 2 months at −20°C, although the purified enzyme in solution did not lose activity (13). The lyophilized protein was resolved by gel filtration chromatography into a high-molecular-weight inactive protein and minor active proteins. The electrophoretic and ultracentrifugation analyses of these proteins suggested that even in the dehydrated state at subzero temperatures, the native xylanase molecules aggregated, leading to a loss of activity. Gomes et al. (99) reported a partial loss of activity in dried culture filtrates of strain DSM 5826 after several months of storage at −20°C but not at 4°C. *H. lanuginosa* xylanase may be useful in testing the stabilization of protein by additives, analyzing the mechanistic basis for preservation of the native structure, and developing strategies of preservation of proteins during freeze-drying.

Native xylanase of a strain of *H. lanuginosa* (*T. lanuginosus*) (245) and recombinant xylanase of strain DSM 5826 (101) were reported to contain a disulfide bond but no free —SH group. The native xylanase of DSM 5826 contained only a

TABLE 7. Some properties of xylanases of thermophilic fungi

Fungus	pI, pH_{Opt}, pH stability	T_{Opt}, stable temp (time)	K_m (mg/ml)	Mol mass (kDa)	% Carbohydrate (wt/wt)	% Hydrolysis of xylan	Reference(s)
Chaetomium thermophile							95
Xylanase I	pH_{Opt} = 4.8–6.0	T_{Opt} = 70°C, stable at 50°C (24 h)	0.55	26	NR[a]	NR	
Xylanase II	pH_{Opt} = 5.4–6.0	T_{Opt} = 60°C, stable at 50°C (24 h)	0.1	7	NR	NR	
Humicola insolens							
Indian isolate	pI = 9.0, pH_{Opt} = 5.5–6.5, stable at 3–10	T_{Opt} = 50–65°C, stable at 50°C	1.33	66	NR	NR	183
Novo Nordisk							77
Xyl I	pI = 9.0, pH_{Opt} = 6–6.5	T_{Opt} = 55–60°C, stable at 50°C	NR	6	NR	12 (LWX)[b]	
Xyl II	pI = 7.7, pH_{Opt} = 6–6.5	T_{Opt} = 55–60°C, stable at 50°C	NR	21	NR	NR	
Humicola lanuginosa							
Japanese isolate	pH_{Opt} = 6.0, stable at 5.0–8.0	T_{Opt} = 65°C, stable at 45–60°C	7.3 (LWX)	21.5		21	142
Indian isolate	pH_{Opt} = 6.0, stable at 6.0–9.0	T_{Opt} = 65°C, stable at 60°C (1 h)	0.9 (LWX)	22.5	1.2	NR	13
Bangladesh isolate (DSM 5826)	pI = 4.1, pH_{Opt} = 4.5–6.5	T_{Opt} = 60–70°C	See text	25.5	Absent	NR	53
ATCC 46882	pI = 3.7, pH_{Opt} = 6.0–6.5; stable at 5.0–9.0	T_{Opt} = 75°C, stable at 60°C (5 h)	NR	25.7	NR	NR	26
Melanocarpus albomyces							199
Xyl IA	pH_{Opt} = 6.6, stable at 5.0–10.0	T_{Opt} = 65°C, stable at 50°C	0.30 (LWX)	38	7	19–38 (LWX, OSX,[b] SBX[b]	
Xyl IIIA	pH_{Opt} = 5.6, stable at 5.0–10.0	T_{Opt} = 65°C, less stable at 50°C	1.69 (LWX)	24	ND[a]	18–27 (LWX, OSX, SBX)	
Paecilomyces varioti	pH_{Opt} = 6.5, stable at 3–10	T_{Opt} = 65°C	2.5 (LWX)	25	4.5	NR	144
Talaromyces byssochlamydoides							111, 112
Xa	pH_{Opt} = 5.5, stable at 3.0–9.0	T_{Opt} = 75°C, $t_{1/2} \approx 5$ min (95°C)		76	36.6	38 (5 days)	
XbI	pH_{Opt} = 4.5, stable at 3.5–8.5	T_{Opt} = 70°C		54	31.5	70 (5 days)	
XbII	pH_{Opt} = 5.0, stable at 3.0–9.0	T_{Opt} = 70°C		45	14.2	75 (5 days)	
Talaromyces emersonii							
Xyl I	pI = 8.9, pH_{Opt} = 2.5	T_{Opt} = 60°C	NR	181 (dimer)	NR	41	253
Xyl II	pI = 5.3, pH_{Opt} = 4.2	T_{Opt} = 78°C	NR	131 (dimer)	NR	27	
Xyl III	pI = 4.2, pH_{Opt} = 3.5	T_{Opt} = 67°C		54.2	NR	NR	
Thermoascus aurantiacus							
New Zealand isolate	pH_{Opt} = 4.5, stable at 4.0–8.5	T_{Opt} = 65°C, stable at 70°C (1 h)		78	5.5		228, 248
C436 (Canadian isolate)	pI = 7.1, pH_{Opt} = 5.1	T_{Opt} = 80°C, stable at 70°C (24 h) and 60°C (97 h)	1.7 (OSX) 1.34 (LWX)	32	NR	>53 (crude)	242
Indian isolate	pH_{Opt} = 5.2	T_{Opt} = 63°C, stable at 70°C (8 h)		31.8	1.0	50 (24 h)	139, 140

[a] NR, not reported; ND, not detectable.
[b] LWX, larch wood xylan; OSX, oat spelt xylan; SBX, sugarcane bagasse xylan.

single, catalytically important cysteine but no intramolecular disulfide bond (53). In view of the postulated important role of disulfide bonding in the thermostability of xylanase (see below), further investigation with fully unfolded xylanase is required to clarify the situation. As has been commonly observed, thermostable proteins exhibit increased stability toward denaturants as well. Xylanase of *H. lanuginosa* was remarkably resistant to denaturation by 8 M urea (245). Heating of xyla-

nase to 60°C for 30 min in the presence of β-mercaptoethanol was necessary to facilitate the reduction of the disulfide bond. The resulting reduced, unfolded protein was functionally inactive and structurally distinct in the presence of urea. The protein had the remarkable ability to refold to its native form upon removal of β-mercaptoethanol, even in the presence of 8 M urea.

Some thermophilic xylanases have been purified, and their primary structures have been determined and crystallized for structural analysis by X-ray crystallography. *T. aurantiacus* xylanase (TAX) (139) contains 301 amino acid residues (150, 184, 231, 255). Its primary structure was highly homologous to that of mesophilic fungal xylanases (150). The substrate-binding site contained glutamate and histidine residues and was lined with negative charges as in other xylanases. Natesh et al. (184) postulated that a disulfide bridge and a preponderance of salt bridges stabilize the protein by holding secondary structures together, thereby contributing to thermostability of the barrel fold. However, when TAX was compared with xylanases from mesophilic sources, there was no correlation between the number of potential salt bridges and increased thermostability (150). Instead, the thermostability of TAX appeared to be due mainly to its well-packed hydrophobic core, favorable interactions of charged side chains with helix dipoles, and the presence of prolines at the N termini of helices that decreased the conformational freedom of the protein molecule. The structure of the xylanase of *T. lanuginosus*, a polypeptide of 225 amino acids with high homology to other xylanases (221), has also been determined (101). The compact, globular protein showed a long cleft that contained the active site. Modeling showed that in the active-site groove, a heptaxylan could be accommodated with the central three sugar rings tightly bound inside the active site. The peripheral sugar rings could assume different orientations and conformations, indicating that the enzyme could also accept xylan chains which are branched at these positions. The thermostability of *T. lanuginosus* xylanase was ascribed to the presence of an extra disulfide bridge, which was absent in the majority of mesophilic xylanases, and to an increased density of charged residues throughout the protein. Studies are being carried out to determine the structure of a xylanase from a thermophilic fungus that was identified as *P. varioti* (83, 144).

α-D-Glucuronidase

Crude culture filtrate protein of *T. aurantiacus* grown on inferior-quality filter paper completely degraded larch wood xylan to produce xylose and 4-*O*-methyl-α-D-glucuronic acid (139). The identification of the enzyme components of the culture filtrate protein led to the purification of an α-D-glucuronidase that released an acidic sugar, glucuronic acid, which is linked by an α(1→2) linkage to xylan (141). The enzyme (EC 3.2.1.139), a single polypeptide of 118 kDa, had optimal activity at pH 4.5 and at 65°C. α-D-Glucuronidase was not as thermostable as xylanase and cellulase purified from the same culture filtrates: at 60°C, more than 50% of the original activity was lost after incubation for 6 h. The three extracellular enzymes of this fungus, xylanase, α-D-glucuronidase, and β-glucosidase, acted together to bring about complete degradation of larch wood xylan.

Polygalacturonase

Since pectin is an important constituent of the plant cell wall, the major pectin-hydrolyzing enzyme, polygalacturonase (E.C. 3.2.1.15), which cleaves the α-1,4 linkages of pectin or polygalacturonic acid, is expected to be commonly produced by thermophilic fungi involved in the decomposition of vegetable matter. However, very few pectin-degrading thermophilic fungal species have been isolated and identified (5). Inamdar (126) screened 40 thermophilic fungal cultures belonging to seven genera by growing them in pectin-containing liquid medium at pH 4.5 (to reduce the degradation of pectin during autoclaving) and assaying the culture filtrates for the presence of polygalacturonase activity. Most of them showed no detectable activity, suggesting that polygalacturonase production may not be species specific but, rather, may depend on the particular isolate. One isolate of *T. aurantiacus* produced more pectinase in medium containing 5% pectin, but the high viscosity of the culture filtrate due to undegraded pectin made purification of the enzyme difficult. A marked enhancement of enzyme production occurred when citrus peel was used compared to purified pectin. Polygalacturonase appeared in the culture fluid before other wall polysaccharide-degrading enzymes (cellulase and xylanase), but its activity fell rapidly whereas that of the other enzymes continued to rise. Polygalacturonase was purified by precipitation of culture filtrate protein by ammonium sulfate and acetone, followed by purification of the protein using ion-exchange chromatography at pH 7.5 and gel filtration. The purified enzyme sedimented as a single homogeneous peak on ultracentrifugation. It had a molecular mass of 37 kDa, a pH optimum of 4.0, and a K_m of 0.4 g of polygalacturonic acid/liter. The polygalacturonase of *T. aurantiacus* appears to be one of the most stable among the polygalacturonases reported. It had a temperature optimum of 65°C and a half-life of 20 min at 55°C. The enzyme mainly produced digalacturonic acid as the end product from hydrolysis of polygalacturonic acid.

Laccase

Laccases (EC 1.10.3.2) are copper-containing enzymes that catalyze the oxidation of phenolic compounds that is accompanied by reduction of oxygen to water. The range of substrates oxidized varies from one laccase to another. The phenolic nucleus is oxidized by a one-electron removal, generating a phenoxy free radical product, which undergoes polymerization. Laccase-like activity has been demonstrated in compost (60). Laccase activity of thermophilic fungi could therefore be important in the polymerization of phenolic substances into humic substances. The gene encoding laccase of *Myceliophthora thermophila* was cloned and expressed in *A. oryzae*, and the recombinant enzyme (r-MtL) was purified from culture broth with a two- to fourfold-higher yield (11 to 19 mg per liter) than that of native (MtL) laccase (28). r-MtL differed from native MtL in three respects: multiplicity of isoforms, higher molecular mass (85 versus 80 kDa), and threefold-higher specific activity. The multiple isoforms contained 40 to 60% carbohydrate and were thought to be due to differential glycosylation. The optimal activity of the enzyme occurred at pH 6.5, and it retained full activity when incubated at 60°C for 20 min.

Laccase from culture filtrates of a thermophilic fungus reported as *Chaetomium thermophilium* was purified by ultrafiltration, anion-exchange chromatography, and affinity chromatography (60). The enzyme was a glycoprotein of 77 kDa. It was stable at a broad pH range from 5 to 10 and at 50°C ($t_{1/2}$ = 12 h). The N-terminal sequence of *C. thermophilium* laccase did not show homology to other fungal laccases. The enzyme polymerized low-molecular-weight water-soluble organic matter fraction isolated from compost into a high-molecular-weight product. These properties suggested that the enzyme

might be involved in the humification process during composting.

Phytase

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases, EC.3.1.3.8) catalyze the hydrolysis of phytic acid (*myo*-inositol hexakisphosphate) to the mono-, di-, tri-, tetra-, and pentaphosphates of *myo*-inositol and inorganic phosphate. Phytic acid is the primary storage form of phosphorus in plant seeds. Thermostable phytases of high catalytic efficiency have commercial prospects because the supplementation of seed-based poultry and pig feed with phytase increases the availability of phosphorus in the feed. Using a phytase-specific probe from *Aspergillus niger*, the *phyA* gene encoding the extracellular phytase from *Thermomyces lanuginosus* was cloned and the recombinant gene product was expressed in *Fusarium venenatum* (27). The heterologous host produced 2 orders of magnitude more enzyme. In contrast to *A. niger* (mesophile) enzyme, the *T. lanuginosus* phytase was active at neutral pH and at 65°C. Differential scanning calorimetry showed that a higher temperature was required to unfold the *T. lanuginosus* phytase (69°C) than to unfold the *A. niger* phytase (60°C).

Phytase genes from several fungi, including the thermophilic species *Myceliophthora thermophila* and *Talaromyces thermophilus*, have been cloned and overexpressed in heterologous hosts (265, 266). The phytases of the aforementioned thermophilic fungi were monomeric glycoproteins with molecular masses of ~63 and 128 kDa, respectively. The phytases released all five equatorial phosphate groups, and the end product was *myo*-inositol 2-monophosphate.

D-Glucosyltransferase

An extracellular D-glucosyltransferase (transglucosidase; EC 2.4.1.24) from *Talaromyces duponti* grown on maltodextrins at 37°C was purified by ammonium sulfate precipitation, hydrophobic interaction chromatography, ion-exchange chromatography, and chromatofocusing (41). The pH and temperature optima of enzyme were 4.5 and 70°C. The enzyme had a molecular mass of 170 kDa and was remarkably thermostable ($t_{1/2}$ = 73 h at 60°C). It may therefore be suitable for the synthesis of α-alkylglucoside (used as a nonionic surfactant) by the transfer of glucosyl moieties from maltooligosaccharide donors to butanol in biphasic medium, since the high temperature can enhance the solubility of the alcohol in the aqueous phase.

In concluding this section, we note that several proteins produced in the growth medium by fungi for assimilative roles are receiving attention from the standpoint of their use in biotechnology. In general, the proteins secreted by thermophilic fungi are intrinsically thermostable. Some wild strains secrete high levels of thermostable proteins which can be used for the determination of the general principles that govern the evolution of thermostable protein.

CELL-ASSOCIATED ENZYMES

In this section we summarize information on enzymes that occur in the cytosol, in the periplasmic space, within the multilayered structure of the cell wall, or on the surface of cells. Only in rare cases is the precise location of the enzyme known. To stimulate interest in thermophilic fungi, the focus will be on any unusual properties and implications thereof, even if the data are rather preliminary.

Enzymes of the Pentose Phosphate Pathway and the TCA Cycle

The first intracellular enzyme studied from a thermophilic fungus was glucose-6-phosphate dehydrogenase (EC 1.1.1.49) of *Penicillium duponti* (43), which is the first enzyme in the pentose phosphate pathway and is involved in the generation of NADPH for biosynthetic reactions. The *P. duponti* enzyme exhibited a sigmoidal saturation curve, suggesting that it may be an allosteric enzyme. The enzyme (Table 8) was very unstable when purified ($t_{1/2}$ = 24 h at 4°C); it was more stable in an impure state (227). Another enzyme of this pathway, 6-phosphogluconate dehydrogenase, was purified from *P. duponti* and *P. notatum* (mesophile) by similar methods (175). The two enzymes had identical molecular masses, but the enzyme from the thermophilic fungus was more resistant to temperature and to urea and acetamide, compounds that break hydrogen bonds. The thermostability of *P. duponti* enzyme was independent of the cultivation temperature. However, aldolase from a thermophilic *Talaromyces* sp. grown at 50 and 30°C had different K_m values, indicating temperature-induced synthesis of isozymes (75).

Eight species of thermophilic fungi were studied to examine the variability in the degree of thermostability in malate dehydrogenase (261). The enzyme (Table 8) from *Humicola lanuginosa* and *Mucor pusillus* were similar in physicochemical properties (259). Although *H. lanuginosa* is the stronger thermophile of the two, its enzyme was less stable than that of *M. pusillus*. Monovalent cations (Na^+, K^+, and NH_4^+) protected both enzymes against heat inactivation without influencing the reaction velocity, indicating that the effect of the cations was possibly due to induced conformational change of the enzyme to a more stable form. The enzymes were also stabilized by citrate.

Trehalase

The substrate of trehalase (EC 3.2.1.28) is the nonreducing disaccharide trehalose, which has the unique property of stabilizing membranes and enzymes against drying and thermal denaturation (62, 68). Therefore, it was thought that this sugar may be present in large amounts in thermophilic fungi. However, trehalose was found in small amounts (less than 5% of the dry weight) in conidia and mycelium of *Thermomyces lanuginosus* (208; G. Bharadwaj and R. Maheshwari, unpublished data), presumably because this fungus has a very active trehalase. In *T. lanuginosus*, trehalase was produced constitutively but was strongly bound to the hyphal wall (200), from which it was solubilized, but only partially, by acetone-butanol treatment of mycelia (201). A partially purified preparation of the enzyme was afforded protection against heat inactivation by unidentified proteins in the mycelial extracts and by an extraneous protein, casein. Subsequently, *T. lanuginosus* trehalase was purified to homogeneity and its properties were compared with those of trehalase from *Neurospora crassa* (30, 202). Trehalases from both sources had acidic pH optima, between 5.0 and 5.5. *T. lanuginosus* trehalase was a monomeric protein of 145 kDa, whereas the *N. crassa* enzyme was a homotetramer with a subunit molecular mass 92 kDa. Both trehalases were glycoproteins, with carbohydrate contents of 20% (*T. lanuginosus*) and 43% (*N. crassa*), were optimally active at 50°C, and exhibited similar thermostability at this temperature ($t_{1/2} > 6$ h). The catalytic efficiency, k_{cat}/K_m, of *N. crassa* trehalase was 1 order of magnitude higher than that of *T. lanuginosus* trehalase. The activation energy for thermal inactivation was twofold higher for the *N. crassa* trehalase (92 kcal/mol) than for the *T. lanuginosus* enzyme (46 kcal/mol). These results

TABLE 8. Properties of some cell-associated enzymes from thermophilic fungi

Enzyme	Source	Mol mass (kDa)	K_m (mM)[d]	Stability ($t_{1/2}$)	E_a (kcal/mol)[b]	Reference(s)
Aminopeptidase	*Talaromyces duponti*	400	5.0 (65°C, pH 7.2) (LPNA)	8 h at 55°C	NR[c]	56
ATP sulfurylase[a]	*Penicillium duponti*	440	0.19 (30°C) (ATP)	5.5 min at 70°C	9.87	216
α-Galactosidase[a]	*Penicillium duponti*	500	NR	Stabilized by intracellular environment	NR	21
β-D-Galactosidase	*Thermomyces lanuginosus*	75	18.2 (lactose)	<1 h at 56°C	NR	87
Glucose-6-phosphate dehydrogenase[a]	*Penicillium duponti*	120	0.16 (25°C) (G-6-P)	~5 min at 55°C	5.5	43, 158
β-D-Glucosidase	*Chaetomium thermophile*	NR	0.075 (PNPG)		NR	151
	Sporotrichum thermophile					
		440	0.5 (ONPG)	15 min at 48°C	NR	170
		40	0.18 (ONPG)	15 min at 47°C	NR	
	Humicola grisea var. *thermoidea*	55	0.08 (PNPF)	12 min at 55°C	NR	197
Invertase	*Thermomyces lanuginosus*	38	12.5	Unstable	8.2	58
Laccase	*Chaetomium thermophilium*	77	0.10 (L-DOPA)	12 h at 50°C	NR	60
Lipoamide dehydrogenase	*Malbranchea pulchella* var. *sulfurea*	102	NR	50 min at 70°C	NR	169
Malate dehydrogenase	*Humicola lanuginosa*	68	4.4 (malate)	14 min at 50°C	4.9	259
	Mucor pusillus	68	5.9 (malate)	60 min at 50°C	5.6	259
Phosphodiesterase	*Talaromyces duponti*	62	NR	100 min at 60°C	NR	89
6-Phosphogluconate dehydrogenase[a]	*Penicillium duponti*	89	NR	6 min at 45°C	10.6	175
Trehalase	*Humicola lanuginosa*	170/145	0.4	~120 min at 60°C	11.4	30, 199, 202
	Humicola grisea var. *thermoidea*					
	Cell surface	580	2.3	NR	NR	275
	Cytosol	360	0.86	NR	NR	52
	Scytalidium thermophilum					133
	Secreted	370	3.58	3 min at 60°C	NR	
	Intracellular	398	2.24	4.5 min at 60°C	NR	
β-D-Xylosidase	*Humicola grisea* var. *thermoidea*	43	0.49 (PNBX)	26 min at 60°C	NR	12

[a] Not purified to homogeneity.
[b] E_a, activation energy.
[c] NR, not reported.
[d] Substrates are given in parentheses. L-DOPA, L-3,4-dihydroxyphenylalanine; G-6-P, glucose-6-phosphate; LPNA, leucine-*p*-nitroanilide; PNPF, *p*-nitrophenyl-β-D-fucoside; PNPG, *p*-nitrophenyl-β-D-glucopyranoside; PNBX, *p*-nitrophenyl-β-D-xylopyranoside.

showed that *N. crassa* trehalase is not only more stable but also a better catalyst than *T. lanuginosus* trehalase.

It is often assumed that enzymes in spores and mycelium are similar if not identical, as for example *N. crassa* trehalase (114). In *Humicola grisea* var. *thermoidea*, trehalases in conidia (275) and mycelium (52) were quite different (Table 8). Whereas conidial trehalase was localized on the surface and was a hexamer composed of three different polypeptides, the mycelial trehalase was cytosolic and a homotrimer. The conidial and mycelial trehalases also differed in their carbohydrate contents (56 and 12%, respectively) and K_m (2.5 and 0.86 mM, respectively) but had similar pH optima (5.5) and temperature optima (60°C). Moreover, both enzymes were stimulated by Ca^{2+}, Co^{2+}, and Mn^{2+} but inhibited by inorganic phosphate, AMP, ADP, and ATP. The addition of calcium disaggregated the mycelial trehalase into three catalytically active but less thermostable forms than the native enzyme, implying a role for self-aggregation of the enzyme molecules in increasing their thermal stability. From the point of view of development, it would be interesting to determine how differences in mycelial and spore trehalases originate. A practical-grade preparation of the conidial trehalase of *H. grisea* var. *thermoidea*, which was free of other glycosidase activities and was stable under storage conditions, may be useful for the quantification of trehalose in biological samples (186).

As noted above, trehalases are bound to the mycelial wall or are present in the cytosol. A thermophilic fungus, identified as *Scytalidium thermophilum* (133), was unusual in that it secreted large amounts of trehalase when grown on starch. Both the intracellular and the extracellular (secreted) forms (Table 8)

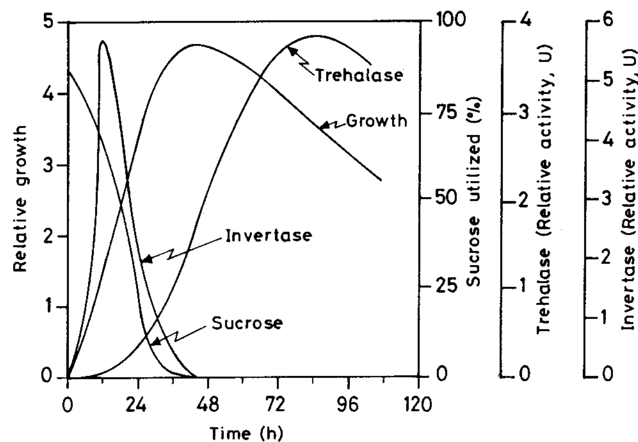

FIG. 4. Distinctive patterns of development of mycelial invertase and trehalase in *Thermomyces lanuginosus* grown in a liquid medium containing sucrose as the carbon source.

were pentamers with similar subunit molecular masses and temperature optima (65 and 60°C), and both contained large amounts of carbohydrate (81 and 51%). Moreover, both enzymes were protected from thermal inactivation by calcium. Unlike the *H. grisea* trehalase, the *S. thermophilum* trehalase was not disaggregated by calcium. The observation that *S. thermophilum* trehalase was associated with the cell wall under one growth condition and secreted under another confirms the dynamic nature of the cell wall (54), whose composition changes in response to environmental conditions and growth state and affects the retention or secretion of wall proteins.

Invertase

Of all the enzymes studied in thermophilic fungi, the behavior of invertase (EC 3.2.1.26) was most unusual. In *T. lanuginosus* (155) and also in *Penicillium duponti* (P. Palanivelu and R. Maheshwari, unpublished data), invertase was an inducible enzyme and its activity was very unstable in cell extracts. By contrast, invertase from mesophilic sources retained activity for long periods under storage conditions. For example, Neuberg and Roberts (185) reported that "toluene autolysates of baker's yeast which had not been purified showed no change of activity after 19 years of storage. The autolysate had been kept in a sealed laboratory bottle on an open shelf; and subjected to the usual fluctuations of temperature and to the action of light." *T. lanuginosus* invertase was stabilized by thiols and inactivated by thiol-modifying compounds, strongly suggesting that it was a thiol protein; its activity depended on the maintenance of a catalytically important sulfhydryl group(s) in the reduced state (58). It was argued that invertase was localized in the hyphal tips, wherein a high ratio of glutathione to oxidized glutathione maintained a reducing intracellular environment favorable for invertase activity (59). Further, the localization of invertase in the hyphal tips explained its peculiar pattern of development, i.e., its inverse relationship to biomass (Fig. 4). Based on indirect evidence, it was postulated that at the early stage of growth, the number of hyphal tips formed per unit mass of mycelium was maximal but at later times the number did not increase in proportion to the biomass (which occurs mainly from cell elongation and wall growth). As a result, invertase activity showed an apparent decline during growth. In contrast, the cell wall-bound trehalase activity in the same fungus increased steadily with time and was highest when growth was completed—a reflection of the amount of wall material formed. Unfortunately, the difficulty of obtaining sufficient starting material (hyphal tips) was exacerbated by the instability of invertase during its purification. Nevertheless, the enzyme was substantially purified by thiol affinity chromatography on an organomercury matrix (58). The purified invertase had pH and temperature optima of 5.6 and 60°C (5-min assay), respectively. The enzyme lost activity in 5 days at 0°C. Surprisingly, unlike in the crude extracts, the inactivated protein was not reactivated by thiol alone but also required a "helper protein" for restoration of activity, suggesting that cellular proteins may enhance and/or stabilize invertase (24, 58). Until more invertases from thermophilic fungi are studied, the observations from *T. lanuginosus* (Table 9) cannot be generalized. Nevertheless, the *T. lanuginosus* example illustrates that activity of unstable intracellular proteins in thermophilic fungi may be optimized in unexpected ways: in this instance, the strategy was its colocalization with substrate transporter in the most strategic region of growing hypha (hyphal tip) and by their substrate-induced synthesis, such that cellular energy was needed for resynthesis of protein(s) only when necessitated by the availability of sucrose in the environment. The suggestion that invertase in *T. lanuginosus* is localized in the hyphal tip needs to be substantiated by immunological techniques. The cloning of the *T. lanuginosus* invertase gene and its heterologous expression will be necessary to understand the unusual behavior of the enzyme in this fungus.

β-Glycosidase

β-Glycosidases (EC 3.2.1.21) catalyze the hydrolysis of alkyl or aryl β-glycosides as well as glycosides containing only carbohydrate residues. Lusis and Becker (151) compared the properties of partially purified preparations of cell-bound and extracellular β-glucosidases from *Chaetomium thermophile* var. *coprophile*. The extracellular enzyme was more stable than the cell-bound enzyme.

Sporotrichum thermophile produced multiple forms of β-glucosidases (Table 8) which differed in molecular mass, pH and temperature optima, thermostability, affinity for β-glycosides, and transglycosylation activity (33, 51, 91, 170). The enzymes are either intracellular or bound to the cell wall, from which some forms are apparently released under conditions that promote cell wall autolysis (91). The functional role of β-glucosi-

TABLE 9. Distinctive features of invertase in *Thermomyces lanuginosus* and *Neurospora crassa*

Property	*Thermomyces lanuginosus* (reference)	*Neurospora crassa* (reference)
Synthesis	Induced by β-fructofuranosides (155)	Constitutive (171)
Distribution	Hyphal tip (59)	Uniformly present along hypha (117)
Relationship to biomass	Activity inversely related to biomass (59)	Activity directly related to biomass (117)
Catabolite repression	Not repressed (154)	Repressed (171)
Stability in vitro	Unstable (almost completely inactivated in 3 days at 0°C) (155)	Stable (> 1 mo at 0°C)
Effect of thiol compounds	Activated and stabilized (59)	None (not reported)

dases is not known. They may be involved in cell wall biosynthesis or remodeling during hyphal elongation. Another possibility is that the cell wall β-glucosidases may combine with cellulase enzymes to form a protein complex that is highly efficient in solubilizing cellulose (91).

An inducible, glycoprotein β-galactosidase from *T. lanuginosus* was purified by fractional salt precipitation, hydrophobic interaction chromatography, and anion-exchange chromatography (87). The enzyme displayed maximum activity at pH 6.7 to 7.2 and was only moderately stable at 56°C, losing all activity in 1 h. At 50°C the enzyme activity in ammonium sulfate precipitates was nearly four times as stable as in the crude extract. The enzyme was dimeric, with a subunit molecular mass of 75 to 80 kDa. The most favorable substrates were *p*- and *o*-nitrophenyl-β-D-galactopyranosides.

Lipoamide Dehydrogenase

Lipoamide dehydrogenase (LD) (EC 1.6.4.3), also called dihydrolipoyl dehydrogenase, is a component of the multienzyme pyruvate dehydrogenase complex. It catalyzes the reduction of NAD^+ with dihydrolipoamide. LD from the thermophilic fungus *Malbranchea pulchella* (Table 8) was purified to homogeneity by a simple three-step procedure consisting of salt precipitation, affinity chromatography, and ion-exchange chromatography (169). *M. pulchella* var. *sulfurea* LD was very similar to the enzyme purified from several other sources. Although the enzyme showed appreciable resistance to thermal denaturation ($t_{1/2}$, 170 min at 65°C), the activity was completely lost in 1 month at 4°C even in concentrated solutions (greater than 2 mg/ml).

ATP Sulfurylase

Renosto et al. (216) compared the properties of the sulfate-activating enzymes ATP sulfurylase (ATP:sulfate adenylyl transferase [EC 2.7.7.4]) and APS kinase (ATP:adenylylsulfate-3′-phosphotransferase [EC 2.7.1.25]) from *Penicillium duponti* with those of the enzyme from the mesophilic fungus *P. chrysogenum*. The enzymes catalyze the first and second reactions in the assimilation of inorganic sulfate: ATP + SO_4^{2-} → PP_i + APS (adenosine-5′-phosphosulfate) and ATP + APS → ADP + PAPS (3′-phosphoadenosine-5′-phosphosulfate). ATP sulfurylase of *P. duponti* (Table 8) had a temperature optimum at 70°C, and that of *P. chrysogenum* was close to 55°C. The thermophile ATP sulfurylase was about 90 times more heat stable (first-order rate constant for denaturation, $k = 0.02$ min^{-1}) than the mesophile enzyme ($k = 1.78$ min^{-1}) and was also more stable in response to urea and to low pH. Both enzymes were hexamers, composed of nearly equal-size subunits (69 kDa), and they had nearly identical amino acid compositions. Antiserum to each enzyme showed cross-reactivity, indicating similar structural determinants. The activation energies of *P. duponti* and *P. chrysogenum* enzymes were 127 and 106 kcal, respectively, both of which are within the usual range.

The APS kinase of *P. duponti* was purified by the same procedure as that used for the *P. chrysogenum* enzyme. However, the *P. duponti* enzyme preparation was not homogeneous and was also not as stable when stored frozen as concentrated solution. At 42°C, the *P. chryosgenum* APS kinase dissociated into inactive subunits but the *P. duponti* enzyme remained intact and active. At 50°C, the specific activity of *P. duponti* ATP sulfurylase was about twofold higher than that of the *P. chrysogenum* enzyme, indicating that the thermophile enzyme was a better catalyst than the mesophile enzyme.

Protein Disulfide Isomerase

Some proteins are active only when their sulfhydryl groups are in the reduced state, and several secretory proteins are active only when folded into three-dimensional structures by correct disulfide bridges. In the cell, protein disulfide isomerase (PDI) (EC 5.3.4.1) in the endoplasmic reticulum facilitates disulfide interchange by shuffling the disulfide bonds to rapidly establish the most thermodynamically stable pairing. The enzyme is useful for refolding and renaturation of scrambled protein in vitro, for basic studies, and for industrial uses. A heat-stable PDI from mycelial extract of *Humicola insolens* was purified in a rapid three-step procedure involving anion-exchange chromatography, concanavalin A affinity chromatography, and reverse-phase high-pressure liquid chromatography (239). Like the yeast and bovine PDI, the *H. insolens* enzyme was a homodimer with a subunit molecular mass of 60 kDa. The cDNA of *H. insolens* PDI was expressed in *Bacillus brevis*, and the recombinant protein secreted into the culture medium was purified to homogeneity in two steps by anion-exchange chromatography and hydrophobic interaction chromatography with a high yield (equivalent to 2.1 g of bovine liver enzyme per liter) (134, 135). The recombinant PDI was 1 kDa smaller because the recombinant protein produced by the bacterial host is not glycosylated. The similar properties of native and recombinant PDIs suggested that glycosylation of the enzyme is not essential for enzymatic activity and stability.

MISCELLANEOUS PROTEINS

The mitochondrial protein cytochrome *c* from several sources has been studied in detail to determine the evolutionary relationships among organisms. The primary structure of cytochrome *c* from *Humicola lanuginosa* was quite similar to that of other organisms, and its sequence in the heme region was quite homologous to that of *Neurospora crassa* (146, 180, 181).

A phosphodiesterase (62 kDa) from *Talaromyces duponti* was purified (89). It displayed optimum activity at pH 6.6 and was activated by cobalt ions. The enzyme retained full activity for 1 h at 50°C. Its $t_{1/2}$ was 100 min at 60°C and 10 min at 70°C.

It is of great interest to know whether the protein-synthesizing machinery of thermophilic fungi has intrinsic thermostability. A study of the effect of temperature on the aminoacylation reaction showed that the maximum activity of the total tRNA synthetase preparation from *H. lanuginosa* was observed at 40°C but that at the temperature for optimum growth (50°C), the aminoacylation activity was only one-half of that at 40°C (132). One species of valine-tRNA synthetase was purified to homogeneity. The purified enzyme was stabilized against inactivation to various degrees by interaction with magnesium ions and the substrates valine, tRNA, and ATP, as well as spermine.

The problem of growing large amounts of mycelia required as the starting material, the difficulty of extracting protein from fungi which possess a multilayered cell wall, and the low specific activities of their enzymes in the crude extracts have, in general, discouraged studies of intracellular enzymes. From the results of a few studies, mostly carried out with partially purified enzyme preparations, the picture that emerges is that intracellular enzymes of thermophilic fungi are less stable than the extracellularly secreted enzymes. This leads to a further consideration that rather than depending on intrinsic thermostability as the primary mechanism in thermal adaptation, eukaryotic thermophily has exploited subtle variations in enzyme structure, catalysis, or mode of regulation.

CONCLUSIONS AND PROSPECTS

Modern studies on thermophilic fungi were stimulated by the prospect of finding fungi capable of secreting high levels of enzymes and of finding novel enzyme variants with high temperature optima and a long "shelf-life," which are desirable characteristics for commercial applications of enzymes. Unfortunately, even before we had a chance to understand or appreciate thermophilic fungi, the questions of their adaptation to high temperatures were brushed aside in favor of practical ends. Consequently, our knowledge of their physiology is very fragmentary. A long-held belief that thermophilic fungi are unable to grow, or grow poorly, at ambient temperatures needs to be reexamined, since some thermophilic fungi exhibit near optimal growth even at room temperature (20 to 30°C). This has led to uncertainty about their minimal temperature of growth. Because of this, thermophilic fungi may be redefined as fungi whose optimum growth temperature is 45°C or above. Although it is unlikely that the temperature range of growth of thermophilic fungi will exceed 32 to 36°C, their latent mesophilic capability may be an advantage, since this would obviate the need for a strict temperature regimen for their industrial cultivation, resulting in savings in operational costs. From the basic point of view, the potential of the same fungus for growth under thermophilic as well as mesophilic conditions poses questions about the biochemical composition of the fungus when grown over a wide temperature range, the rapidity with which metabolism is reorganized, and the mechanisms involved in metabolic adjustments. The question why thermophilic fungi have not been able to breach the upper temperature limit of growth of 60 to 62°C is still not resolved.

Because thermophilic fungi occur in terrestrial habitats which are heterogeneous in terms of temperature and the types and concentrations of nutrients, chemicals, gases, water activity, competing species, and other variables, they may be able to adapt to several factors besides just high temperature. From this perspective, research should extend to their nutrient uptake systems, their ability to utilize mixed substrates, the nature and concentrations of their intracellular ions and osmolytes, and their effects on enzyme function. In addition, the composition of membrane lipids and of membrane-bound enzymes, the composition of their respiratory chain, and their energy production when grown under thermophilic and mesophilic conditions are other aspects that should be investigated. Only when several species are studied and the results are compared will we be able to determine which of the observed responses is an adaptive strategy for thermophilic growth. In general, our knowledge of the above areas of fungal physiology is very limited. Perhaps as thermophilic fungi become better known, a fascination for these organisms will lead to basic studies and their physiology and biochemistry will be better understood. The ease of their isolation and maintenance of cultures, the reduced risk of contamination of thermophilic cultures, their simple nutrition and rapid growth, and the formation of homogeneous mycelia in suspension cultures are desirable features for many types of experimental studies.

Although thermophilic fungi have long been known to be involved in composting and humification, the mechanisms involved in the accelerated decomposition of biomass are not well understood. However, their role in decomposition of vegetable matter suggests that thermophilic fungi may be good sources of a battery of purified enzymes that are capable of disassembling plant cell walls, which are required as tools by plant biologists for elucidation of cell wall architecture.

The extracellular enzymes of thermophilic fungi are appreciably thermostable, but less so than those of hyperthermophilic archaea (2). Currently, enzymes from hyperthermophiles are being favored, with prospects in biotechnology. A realistic assessment of the degree of thermostability needed for industrial applications of enzymes is required. Since flexibility of proteins is essential for catalysis, the enzymes from hyperthermophiles will have optimal conformational flexibility at the temperatures at which they are adapted to grow (80°C or above) but may become too rigid and have low catalytic rates at the operational temperatures which in most situations range from 50 to 65°C. Therefore, in most situations, enzymes having moderately high temperature optima and thermostability, as has been observed for extracellular proteins of thermophilic fungi, may be better suited than enzymes from hyperthermophiles. In basic studies, enzymes of thermophilic fungi (eukaryotes) will provide additional material for comparative analysis of the extent of modification in kinetic properties of enzymes vis-à-vis their thermal stability. Some thermophilic fungi have yielded crystalline preparations of thermostable proteins that are contributing to structural information on the rules that determine thermostability of proteins and the critical groups involved in catalysis. This information will be essential for designing alteration in the genes encoding enzymes for specific applications.

The available information suggests that the thermostability of intracellular enzymes of thermophilic fungi is not appreciably different from that of mesophilic fungi. In fact, instances of unstable enzymes in thermophilic fungi have come to light. Although the studies so far have been preliminary, there are indications that eukaryotic thermophily involves a repertoire of mechanisms of stabilization of enzymes, with different mechanisms operating for different proteins: intrinsic thermostability and stabilization by ions, other cellular proteins including chaperonin molecules, self-aggregation, and possibly covalent or noncovalent associations with the polymeric constituents of the cell wall. The activity of certain sensitive enzymes may be optimized by their induced synthesis only when needed, by their placement in the most strategic location of the hypha, and by their rapid resynthesis. In this regard, the finding in a thermophilic fungus of a novel invertase—an enzyme that has figured prominently in the development of biochemistry—suggests that investigations may reveal other examples of enzymes that are regulated differently in thermophilic fungi. Such examples pose challenging questions about the significance of the observed difference, including whether the observed differences are adaptive responses to thermophily or to conditions that normally prevail in the habitat. Cloning of some genes of thermophilic fungi and their heterologous expression have provided answers to questions concerning their biosynthesis, structure, and role of carbohydrate moieties in proteins. Heterologous expression will undoubtedly be extended to other enzymes, which are difficult to obtain in sufficient amounts from the native strain. Some thermophilic fungi produce high levels of extracellular enzymes that are of interest in biotechnology. Knowledge of the structure and function of transcription control regions of the genes encoding these proteins may be used to construct recombinant genes for their overexpression.

Because thermophilic fungi are found in a variety of habitats from which they are easily isolated, many investigators have been prompted to use their own strains in enzyme studies. This is of course welcome, since studies with different isolates are required to obtain information on intraspecies differences in the properties of the same enzyme. However, in some cases the marked differences observed in properties of enzymes in "strains" from different geographical backgrounds have raised doubts about whether the taxon was, in fact, correctly identi-

fied. A comparison of the properties of an enzyme from two or more strains by the same method will be necessary to determine if high variability is a characteristic feature of thermophilic fungi. If so, investigations of the mechanisms generating variation may provide opportunities for fundamental discoveries. Finally, as has been said repeatedly, progress in science depends on prior investigations. It is therefore important that scientists share cultures of thermophilic fungi and cloned genes.

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