Degradation of Starchy Endosperm Cell Walls in Nongerminating Sterilized Barley by Fungi

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Strains of fungi from different origins, including isolates of the natural microflora of barley, were screened for their ability to modify barley starchy endosperm cell walls in situ. In an initial step, fungi were selected that degrade the major component of the cell walls, that is, (1→3),(1→4)-*β*-D-glucan, in vitro on artificial media. Nongerminating, sterilized barley, obtained by *γ*-irradiation, was inoculated with such fungi and subjected to solid state fermentation under conditions resembling those of a traditional malting process. For some strains of fungi, a clear correlation between the production of endo-*β*-glucanase and the friability of the treated kernels was found. Image analysis of Calcofluor stained longitudinal sections of barley kernels fermented with the endo-*β*-glucanase producing strains showed that starchy endosperm cell walls were modified. As malt quality is inversely related to its (1→3),(1→4)-*β*-D-glucan content, the selected strains have high potential to be used as starter cultures during malt production, contributing to the processing quality of the final product.

Keywords: *Barley malt quality; cell wall modification; starter culture selection*

INTRODUCTION

Important criteria for malt quality in terms of brewing performance are that extract yield is high and that no problems occur during the production of this extract. This implies that malt modification is high and homogeneous and ideally is independent of the quality of the raw material used. Variation in malt quality therefore in most cases can be reduced to an imbalance of the plant endogenous enzymes that are responsible for the modification process, especially for the modification of the starchy endosperm cell walls.

Barley starchy endosperm cells have relatively thin walls with (1→3),(1→4)-*β*-D-glucan (hereafter referred to as *β*-glucan) (∼71%) and arabinoxylan (∼19%) as major polysaccharide components (*1, 2*). Although the exact molecular organization of the polymers in the cell wall is unknown, the current belief is that they are organized as a multicomponent amorphous layer in which a network of (quantitatively less important) cellulose microfibrils and structural proteins is embedded (*3, 4*).

During barley germination, the degradation of the cell walls is the first step in the sequence of the hydrolytic processes, generally referred to as modification. The nature of the enzyme activities that initially release the cell wall polysaccharides from the matrix is not precisely known (*4*). Released *β*-glucan is subsequently degraded by endo-1,3(4)-*β*-glucanases (hereafter referred to as *β*-glucanases), and the oligosaccharides produced are degraded to glucose by *β*-glucosidases. Endo-1,4-*β*-xylanases (hereafter referred to as xylanases) and α-arabinofuranosidases hydrolyze arabinoxylan (*3*).

The occurrence of unmodified regions in the starchy endosperm of malt is related to a high polymeric *β*-glucan content and is influenced both by the *β*-glucan content of barley and by the activity of *β*-glucan degrading enzymes during malting (*5*). Because of the asymmetric shape and high molecular weight (150000−300000) of these molecules and the possible occurrence of hydrogen bridges between *β*-glucan chains, the presence of excessive residual *β*-glucan in brewing may lead to viscous solutions and formation of aggregates with other mash components (*4*). The problems caused by *β*-glucan are summarized by Bamforth (*6*): reduced recovery of extract by impeded enzyme access and reduced rates of lautering, reduced filter runs, and formation of gels and hazes.

The main objective of the present study was to select microorganisms that during their development on barley effect cell wall modification by secretion of cell wall degrading enzymes. From literature data, it is clear that, although barley physiology is of utmost importance for malt quality, the impact of the natural microbial population of barley on malt quality aspects cannot be ignored (*7*). Members of the field microflora release *β*-glucan from isolated cell wall material (*8*). In vitro assays showed that isolated fungal contaminants degrade arabinoxylan and carboxymethylcellulose (*9, 10*). Hoy et al. (*11*) even claimed that most cellulase activity in germinating barley is of microbial origin. Furthermore, it has been suggested that members of the barley microflora contribute to *β*-glucan degradation during malt production (*12, 13*). The actual role and the

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potential significance of microbial enzymes in the malt modification process are, however, not clear at present.

MATERIALS AND METHODS

Chemicals and Media. Azoxylan (oat spelt), β-glucan (barley, viscosity = 20−30 cSt) as well as β-glucazyme, amylazyme, and xylazyme AX tablets were obtained from Megazyme (Bray, Ireland). Potato dextrose agar (PDA) was from Unipath (Hampshire, U.K.) and agar from Life Technologies (Paisley, U.K.). Oxgall, soluble starch, and yeast nitrogen base (YNB) were from Difco Laboratories (Detroit, MI). Calcofluor white, chloramphenicol, Congo red, Fast green, primulin, and tris(hydroxymethyl)aminomethane (TRIS) were obtained from Sigma-Aldrich (Bornem, Belgium). Tryptone soy agar supplemented with 100 ppm of pimaricine (TSAp) and oxytetracycline gentamicine yeast extract agar (OGYE) were prepared as described by Van Campenhout et al. (*14*). The preparation of malt salt agar (MSA) and pentachloronitrobenzene agar (PCNB) was performed according to the method of Rabie et al. (*15*).

γ-Irradiation of Barley. On a laboratory scale (200 g), γ-irradiation was carried out in a Gammator M 34-3 source (Radiation Machinery Corp.), whereas 1 kg samples were treated in a Gammacell 2200 (Caric-Mediris, Fleurus, Belgium).

Fungal Strains: Isolation, Cultivation, and Preparation of Inoculum. Fungi were isolated from barley and malting barley samples (1994, French and German harvests). Soil fungi capable of invading barley kernels were isolated from kernels that had first been sterilized (10 kGy) and then incubated in moistened nonsterile soil for 5 days at 18 °C. The isolates were identified to genus level using keys described by Pitt and Hocking (*16*). Additional fungal strains from our laboratory culture collection were included in the study. The strains were maintained as stock cultures at 4 °C on PDA slants.

Fungal inoculum was prepared by flooding a 7-day-old culture, grown at 28 °C on a PDA slant in the dark, with sterile physiological saline [0.9% (w/v) sodium chloride]. The mycelium was gently rubbed with a sterile spatula, the suspension was washed and centrifuged (1600*g*, 15 min) twice, and the pellet was resuspended in sterile physiological saline. The cell density of the suspension was determined microscopically using a Thoma counting chamber. For inoculation of whole barley kernels, $\sim 10^4$ spores per gram of dry weight (dw) barley were used. For nonsporulating strains, mycelial fragments were inoculated.

Detection of Enzyme Production on Artificial Media. The method of Farkaš et al. (*17*) with some modifications was used for the detection of β-glucanase, xylanase, and amylase production. Agar plates containing 0.67% (w/v) YNB, 1% w/v oxgall, 2% (w/v) agar, and 0.2% (w/v) β-glucan, azoxylan, or starch were inoculated with 10 μL of a suspension of the fungal strain. The inoculated plates were incubated for 72 h at 28 °C. After incubation, the growth media containing β-glucan were held for 3 h at 50 °C. β-Glucanase activity could be observed after the remaining β-glucan had been stained with 1% (w/v) Congo red solution and the medium rinsed twice with 1.0 M sodium chloride. Xylanase activity was detected as the appearance of a clearing zone around the colony. Starch degradation was observed after the growth media had been treated with iodine vapors.

Solid State Fermentation of Nongerminating Sterilized Barley Kernels. Strains with a β-glucan degrading activity in the agar plate test were inoculated on nongerminating sterilized barley (150 g) that was first steeped aseptically during 24 h at 18 °C in sterile deionized water to a moisture content of 40.7 (±1.0)%. The inoculum was added to the steeped barley at the end of the steeping process. After draining, the barley was fermented in an Erlenmeyer flask (1 L) for 5 days at 18 °C and kilned in a Joe White micromalting unit (Perth, Australia) using seven temperature steps (3 h at 62 °C, 2 h at 65 °C, 2 h at 68 °C, 2 h at 73 °C, 1 h at 78 °C, 2 h at 80 °C, and 6 h at 83 °C).

Measurement of Enzymatic Activities in Extracts of Barley and "Malt". To determine the β-glucanase activity, the method of McCleary and Shameer (*18*) was used with some modifications. Ground sample (1.0 g, Waring Blendor) was suspended in 10.0 mL of extraction buffer (50 mM sodium acetate, pH 4.2). The suspension was shaken for 15 min at room temperature and centrifuged (1250*g*, 10 min, 10 °C). The β-glucanase activity was measured by incubating 1.0 mL of the appropriately diluted supernatant with a β-glucazyme tablet at 40 °C. Addition of 10.0 mL of 2% (w/v) TRIS after exactly 10 min of incubation and vigorous vortex stirring terminated the reaction. The slurry was filtered through a Whatman No. 1 filter, and the extinction was measured at 590 nm against a control. The α-amylase activity was measured in a similar way with amylazyme tablets and by making use of 100 mM sodium maleate (pH 6.0) containing 5 mM calcium chloride as extraction buffer. The xylanase activity was determined according to the method of McCleary (*19*) with some modifications. Ground sample (1.0 g, Waring Blendor) was suspended in 5.0 mL of extraction buffer (25 mM sodium acetate, pH 4.7). The suspension was shaken for 20 min at room temperature and centrifuged (2800*g*, 15 min, 10 °C). The xylanase activity was measured by incubating 1.0 mL of appropriately diluted supernatant with a xylazyme AX tablet at 50 °C. Addition of 10.0 mL of 1% (w/v) TRIS after exactly 60 min of incubation and vigorous vortex stirring terminated the reaction. The slurry was filtered through a Whatman No. 1 filter, and the extinction was measured at 590 nm against a control. Enzyme activities are expressed as extinction values per gram of barley or "malt" (E_{590}/g).

Barley and "Malt" Analyses. Barley was analyzed for microbial populations [colony-forming units (CFU) per gram] by serial dilution plating using TSAp and OGYA as enumeration media for bacteria and yeasts, respectively. Grain infection by filamentous fungi was assessed by directly plating 100 grains, surface disinfected with 80% (v/v) ethanol and rinsed twice with sterile deionized water, onto MSA for storage fungi, PCNB for *Fusarium*, and PDA supplemented with 100 ppm of chloramphenicol for field fungi. Percentages of infected grains were determined by visual inspection after incubation. Germinative capacity and germinative energy (4.0 and 8.0 mL) were tested using the hydrogen peroxide and the BRF methods, respectively (*20*). Germinative power was measured using the Schönjahn method (*3*). The Calcofluor method (*20*) was used to determine the modification of the kernels. The Calcofluor apparatus (Haffmans, Venlo, The Netherlands) was calibrated with standard malt. The friability of the "malt" was determined according to ref *20*. The moisture content was determined by loss of weight upon drying for 150 min at 150 °C.

Microscopic Analysis of Longitudinal Sections of "Malt". Longitudinal sections of "malt" kernels, stained with Calcofluor for β-glucan and Fast green as counter stain, were examined in more detail with an epifluorescence microscope (Carl Zeiss, Jena, Germany) (excitation at 395−440 nm, barrier filter at 470 nm). Fungal mycelium in longitudinal sections of inoculated barley was detected after 15 min of staining with diluted primulin (1:1000) (*21*).

Statistical Analyses. The enzyme production screening data were analyzed using nonlinear regression techniques (CARDS). Experiments were carried out in triplicate. Average results and 95% confidence intervals (Students' *t*) are presented.

RESULTS AND DISCUSSION

Selection and Screening of Fungal Strains. We have focused the search on microorganisms that secrete adequate cell wall hydrolyzing enzymes in the barley endosperm. A collection of 220 strains of fungi was screened for β-glucan and xylan degrading activities because it was anticipated that these two enzymatic activities are essential for barley endosperm cell wall modification. The selection was performed by testing

Table 1. In Vitro Degradation of β-Glucan, Xylan, and Starch by Fungal Strains

genus		source	β-glucan	xylan	starch
Aspergillus	I	barley	++[a]	++	++
	II	barley	++	++	++
	III	barley	0[b]	++	++
	IV	barley	0	++	+
	V	barley	0	++	++
	VI	barley	0	++	++
	VII	barley	+[c]	++	++
	VIII	barley	0	++	++
	IX	barley	0	++	++
	X	barley	0	++	+
	XI	soil	+	++	++
	XII	soil	0	+	++
	XIII	LCC[d]	++	++	++
	XIV	LCC	++	++	++
	XV	LCC	0	++	++
	XVI	LCC	++	++	++
	XVII	LCC	0	++	++
	XVIII	LCC	++	++	++
Geotrichum	I	barley	0	0	0
	II	barley	0	0	0
	III	barley	0	0	0
	IV	LCC	++	0	0
	V	LCC	++	0	0
Mucor	I	barley	++	0	++
	II	soil	++	0	++
	III	soil	+	+	++
Rhizopus	I	barley	++	0	++
	II	LCC	++	0	++
	III	LCC	0	0	0
	IV	LCC	+	0	++
	V	LCC	0	0	++
	VI	LCC	++	0	++
	VII	LCC	++	0	++
	VIII	LCC	+	0	++

[a] ++, intensive degradation. [b] 0, no degradation. [c] +, degradation. [d] Laboratory culture collection.

enzyme production on artificial media, containing β-glucan or xylan as the sole source of carbon. The ability to degrade starch, the most abundant carbon source in barley, was also evaluated. The screening results allowed us to acquire insight into the enzymatic potential of the barley mycoflora. A high number of isolates were able to degrade β-glucan and xylan on an artificial medium: 56% degraded β-glucan and 77% degraded xylan, whereas 39% of the tested strains degraded both substrates. The finding that xylanase production was more common than β-glucanase production is in agreement with the conclusions of Flannigan (*9*) and Flannigan and Sellars (*10*), who earlier found, in in vitro assays, that among fungal isolates from barley belonging to 23 different genera, arabinoxylan degradation was more common than carboxymethylcellulose degradation. These authors concluded that xylanase activity may be of greater importance than cellulase activity in making sugars available for growth on the husk.

Because the objective of our work is to select fungal strains that are potentially useful as starter cultures in malt production, we retained in a next step only those strains belonging to genera that are used in mold-fermented foods of plant origin. These include genera that are used as starter cultures in Asian cereal fermentation technologies such as *Aspergillus*, *Mucor*, and *Rhizopus* (*22*) or as biocontrol agents in malting such as *Geotrichum* (*23*, *24*). Several strains of these four genera from our culture collection produced β-glucanolytic enzymes (Table 1). All *Aspergillus* strains and one *Mucor* strain also degraded xylan. All *Aspergillus* and *Mucor* strains, all but one *Rhizopus* strains, but no *Geotrichum* strains used starch as a carbon source. β-Glucan degrading *Aspergillus*, *Geotrichum*, *Mucor*, and *Rhizopus* strains were then subjected to an experiment designed to select for the ability of the strains to cause modification of the endosperm cell walls of barley kernels. For this purpose sterile barley kernels with native cellular structures but not producing endogenous cell wall hydrolases were required as a substrate. We attempted to achieve this by γ-irradiation and studied the effect of γ-irradiation on barley germination and microbial load.

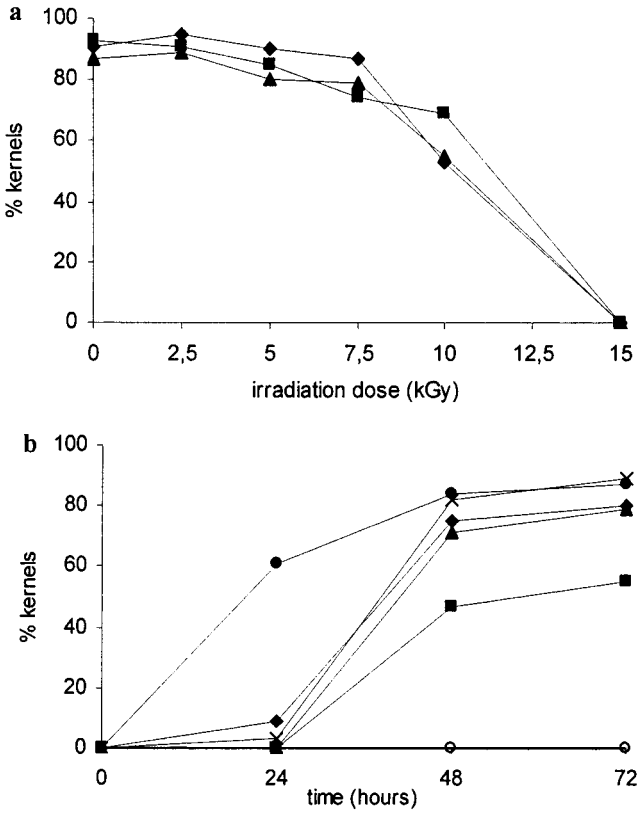

Figure 1. Influence of γ-irradiation on barley germination: (a) germinative energy (◆), germinative capacity (▲), and germinative power (■) of barley as a function of the irradiation dosage; (b) germinative power of barley irradiated with dosages of 0 (●), 2.5 (×), 5 (◆), 7.5 (▲), 10 (■), or 15 (○) kGy as a function of the incubation time.

Influence of γ-Irradiation on Germination and Microbial Contamination of Barley. On a laboratory scale, the natural microflora of various barley samples was completely destroyed at a dosage of 10 kGy, as measured by plating whole kernels and homogenates of the irradiated kernels. This is in good agreement with a report by Ramakrishna et al. (*25*), who found that 4 kGy eliminated most *Alternaria*, *Fusarium*, and *Epicoccum* species and dosages up to 12 kGy killed most *Bacillus* species, yeasts, and *Aureobasidium pullulans*. Regarding germinative properties, an irradiation dosage of 10 kGy or higher resulted in a large reduction of germinative energy, power, and capacity (Figure 1a). Germination was also retarded when barley was irradiated (Figure 1b), although irradiation dosages up to 7.5 kGy had only a small effect on the germinative energy measured after 3 days. Basson and de Villiers (*26*) studied the effect of dosages up to 7.5 kGy and demonstrated that barley germinative energy values were significantly lowered at a dosage of 7.5 kGy.

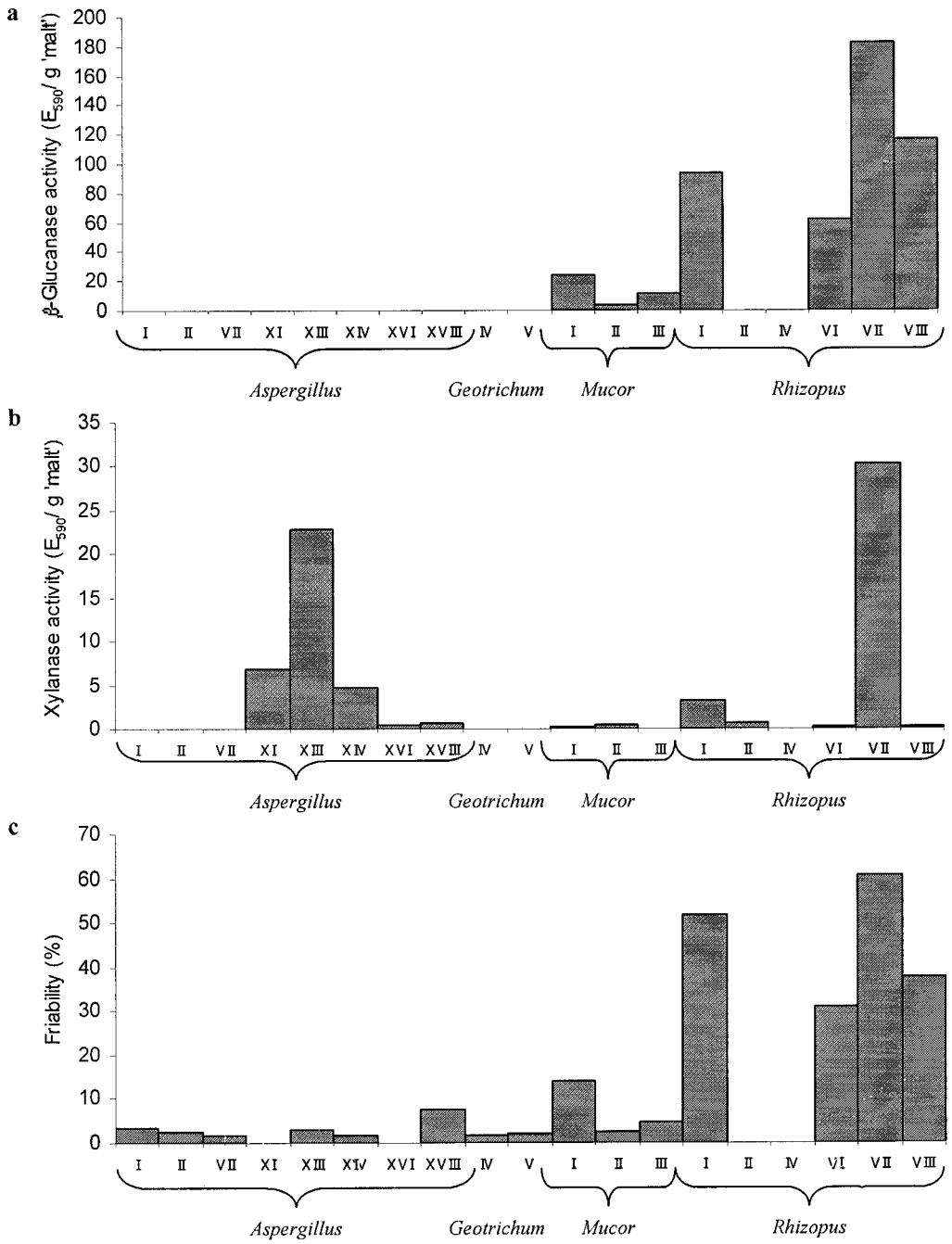

Figure 2. β-Glucanase (a) and xylanase (b) activities (E_{590}/g) in extracts of "malt" and friability (c) (percent) of "malt" obtained after fermentation of nongerminating sterilized barley with various fungal strains.

Nongerminating barley was obtained on a laboratory scale after irradiation with a dosage of 15 kGy, whereas on a larger scale a dosage of 20 kGy was required. This treatment provided sterile barley that did not produce endogenous enzymes under conditions resembling those of a malting process. The cellular structure of the kernel tissue was indistinguishable from that of untreated barley upon microscopic analysis of kernel sections.

Effect of Selected Strains on Cell Wall Modification of Irradiated Nongerminating Barley. The ability of the selected strains to secrete enzymes during their growth on steeped irradiated nongerminating barley under conditions resembling those of a traditional malting process and the effects of such enzymes on the endosperm cell walls were examined. The β-glucanase and xylanase activities obtained after fermentation of nongerminating sterilized barley with various fungal strains are presented in parts a and b, respectively, of Figure 2. Average values for enzymatic activities and friability of noninoculated control samples [β-glucanase, 0.22 (E_{590}/g); xylanase, 0.13 (E_{590}/g); and friability, 11.6 (%)] were subtracted. Furthermore, the measured activities are the net result of enzyme production by the fungi during their development on the grains and of enzyme inactivation during subsequent kilning. Only a limited number of strains with β-glucan degrading activity on an artificial medium produced a significant level of this enzyme during growth on barley kernels. The highest level of β-glucanase was produced by *Rhizopus* strains, but it was also produced by *Mucor* strains. Several *Mucor* and *Rhizopus* strains produced xylanase. After fermentation of barley with *Aspergillus* strains, xylanase production was measured for a num-

Table 2. Enzymatic Activities, Friability, and Calcofluor Modification of "Malt" Obtained after Fermentation of Nongerminating Sterilized Barley with *Rhizopus* Strains I, VI, VII, and VIII and of Noninoculated Control Samples

	Rhizopus strain				
	I	VI	VII	VIII	control
β-glucanase activity (E_{590}/g)	90.0 (±8.3)[a]	70.0 (±2.5)	153.3 (±20.7)	121.7 (±12.6)	0.2 (±0.1)
xylanase activity (E_{590}/g)	5.1 (±1.8)	1.7 (±1.1)	20.1 (±7.3)	1.9 (±1.3)	0.1 (±0.0)
friability (%)	62.1 (±1.3)	40.2 (±2.3)	63.7 (±7.4)	44.3 (±4.8)	11.6 (±1.9)
Calcofluor modification (%)	74.9 (±5.2)	63.5 (±4.1)	82.4 (±2.0)	74.1 (±2.5)	29.5 (±2.3)
kernels 95−100% modified (%)	50 (±4)	29 (±10)	53 (±13)	40 (±9)	0 (±0)

[a] 95% confidence interval (Student's *t*).

ber of strains. Neither β-glucanase nor xylanase activities were produced by the tested *Geotrichum* strains.

As cell wall modification during regular malting results in a decrease of the grain hardness, the final product friability is regarded as an index for modification (*20*). A clear correlation between the malt friability and the microbial production of β-glucanase but not of xylanase was found (Figure 2). This correlation was confirmed by statistical analysis. This increase in friability shows that the cell wall material was degraded in the absence of plant enzymes.

The enzymatic activities resulting in significant increase in modification were produced by *Rhizopus* strains I, VI, VII, and VIII. These strains also showed the highest β-glucanase production during development on barley. Although strains I and VII did not degrade xylan on an artificial medium, they produced xylanase when grown on barley. Average results and standard deviations for enzymatic activities and friability of the samples obtained after fermentation by *Rhizopus* strains I, VI, VII, and VIII are presented in Table 2. Fungi of the order of the *Mucorales*, to which *Rhizopus* strains belong, are well suited for the manufacture of a number of fermented foods by their rapid growth and by the abundant production of enzymes (*27*). Traditional foods fermented with *Mucorales* species that are important in food supply are tempe, oncom, and tofu (*22*). One of the *Rhizopus* strains (I) is an isolate of the natural barley microflora. The results obtained for this strain therefore suggest that the natural microbial population may have an influence on the malt modification process. However, we assume that, under normal conditions of malt production, the levels of these naturally occurring contaminants on barley are too low to result in any significant effects.

Although friability does provide indirect evidence of cell wall degradation, a histochemical technique based on the specific interaction of the fluorescent dye Calcofluor with β-glucan molecules with a molecular weight >10000 (*28*) offers a more direct approach. Image analysis of stained longitudinal sections allows detection and quantification of areas in the endosperm that still contain high molecular weight β-glucan, indicating the presence of intact cell walls. As Calcofluor modification results show that microbial enzymes (Table 2) degraded endosperm cell walls, further evidence for the modification of the endosperm cell walls was obtained. On the basis of these measurements, the extent of modification obtained seems to be strain dependent. However, with all four inoculants, the complete degradation of the β-glucan component of the endosperm cell walls was observed in longitudinal sections of a high percentage of kernels. The modification value obtained for the noninoculated sample was unexpected because the irradiated barley does not produce endogenous enzymes, but can be explained by the standard calibration procedure for the Calcofluor apparatus using regular malt. Most likely, the threshold value selected by this procedure is not suitable to evaluate nonmodified barley cross sections. Detailed microscopic analysis of Calcofluor and Fast green stained longitudinal sections confirmed that β-glucan was released from the cell wall matrix and degraded as endosperm cell walls (blue) were not detected in parts of the "malt" endosperm section. Examples of kernels that were fermented by *Rhizopus* VII are shown in Figure 3. Because the experiments were conducted with nongerminating barley, which could not produce endogenous enzymes, it must be concluded from this analysis that the fungal enzymes caused cell wall degradation.

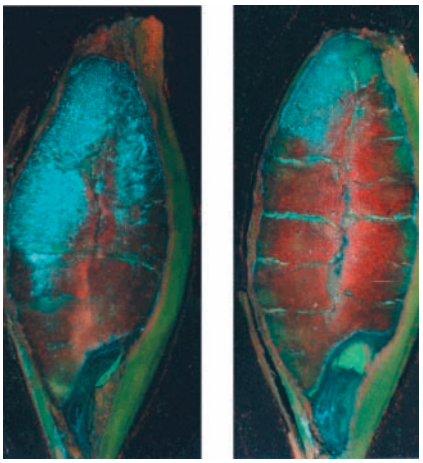

Figure 3. Photomicrographs of longitudinal sections of "malt" obtained after fermentation of nongerminating sterilized barley with a *Rhizopus* strain VII (magnification 10.3×). Stainings: endosperm cell walls, blue; endosperm protein, green; starch, red. Cracks were caused by sample preparation.

Histochemical Analysis of Fungal Colonization of Barley Endosperm. Fungal mycelium was detected in the endosperm of the "malt" kernels after staining with primulin, a chitin binding fluorescent dye. For regular barley, it is assumed that hyphae of natural fungal contaminants occur only in the outer layers of the grain, that is, in the lemma and palea, and within the pericarp layer of the caryopsis (*29*). The presence of *Rhizopus* mycelium in the endosperm in the current work should therefore be confirmed by a more specific method such as immunohistochemical staining. However, if we assume that the observed mycelium in the endosperm originates from the inoculated strain, the fungus is able to invade barley kernels and deliver enzymes that degrade the cell walls in the endosperm. In tempe production also, it is suggested that diffusion of β-glucanase and xylanase in the soybeans is related to the invasion of hyphae of *Rhizopus oligosporus* in the substrate (*30*). At this point, we cannot exclude the possibility that the γ-irradiation of barley has caused

tissue damage and allowed infection in the endosperm. Also, irradiation may have eliminated the ability of the barley to respond to fungal invasion by induced defense mechanisms, as well as potential antagonisms that are possibly present in the natural microflora. On the other hand, it seems that antimicrobial compounds already present in barley kernels (such as thaumatin-like proteins and hordatine) (*31*) did not inhibit the inoculated strains. Other points that remain to be clarified are whether fungal invasion of the barley endosperm occurs through pre-existing lesions in the outer layers and whether intact kernels can also be infected.

Conclusions. Enzymes produced during the development of selected fungal strains belonging to the order of the *Mucorales* on nongerminating sterilized barley cause endosperm cell wall modification. It was demonstrated that, under conditions resembling those of a malting process, the microbial enzymes were delivered in the barley endosperm and that the major cell wall polymer, β-glucan, was degraded in the absence of plant enzymes. Therefore, it can be concluded that in situ fermentation of barley kernels by selected microorganisms leads to desirable changes and significant alteration in specific quality parameters. Histochemical analysis revealed that the fungi were capable of invading the nongerminating sterilized barley kernels. To evaluate the potential of these strains as starter cultures to enhance endosperm modification in malt production, the interaction with the barley physiological processes and the natural microflora need to be studied. Also, the effect of the use of starter cultures on malt quality aspects other than cell wall modification needs to be considered.

ABBREVIATIONS USED

CFU, colony-forming units; dw, dry weight; MSA, malt salt agar; OGYE, oxytetracycline gentamicine yeast extract agar; PCNB, pentachloronitrobenzene agar; PDA, potato dextrose agar; TRIS, tris(hydroxymethyl)aminomethane; TSAp, tryptone soy agar supplemented with 100 ppm of pimaricine; YNB, yeast nitrogen base.

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