J Ind Microbiol Biotechnol (2006) 33:953–966
DOI 10.1007/s10295-006-0150-z

ORIGINAL PAPER

Yeasts in an industrial malting ecosystem

A. Laitila · A. Wilhelmson · E. Kotaviita · J. Olkku · S. Home · R. Juvonen

Received: 13 February 2006 / Accepted: 17 May 2006 / Published online: 7 June 2006

Abstract The malting ecosystem consists of two components: the germinating cereal grains and the complex microbial community. Yeasts and yeast-like fungi are an important part of this ecosystem, but the composition and the effects of this microbial group have been largely unknown. In this study we surveyed the development of yeasts and yeast-like fungi in four industrial scale malting processes. A total of 136 malting process samples were collected and examined for the presence of yeasts growing at 15, 25 and 37°C. More than 700 colonies were isolated and characterized. The isolates were discriminated by PCR-fingerprinting with microsatellite primer (M13). Yeasts representing different fingerprint types were identified by sequence analysis of the D1/D2 domain of the 26S rRNA gene. Furthermore, identified yeasts were screened for the production of α-amylase, β-glucanase, cellulase and xylanase. A numerous and diverse yeast community consisting of both ascomycetous (25) and basidiomycetous (18) species was detected in the various stages of the malting process. The most frequently isolated ascomycetous yeasts belonged to the genera *Candida*, *Clavispora*, *Galactomyces*, *Hanseniaspora*, *Issatchenkia*, *Pichia*, *Saccharomyces* and *Williopsis* and the basidiomycetous yeasts to *Bulleromyces*, *Filobasidium*, *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon*. In addition, two ascomycetous yeast-like fungi (black yeasts) belonging to the genera *Aureobasidium* and *Exophiala* were commonly detected. Yeasts and yeast-like fungi produced extracellular hydrolytic enzymes with a potentially positive contribution to the malt enzyme spectrum. Knowledge of the microbial diversity provides a basis for microflora management and understanding of the role of microbes in the cereal germination process.

Keywords Barley · Malting · Yeast · Diversity · Enzyme

A. Laitila (✉) · A. Wilhelmson · S. Home · R. Juvonen
VTT Technical Research Centre of Finland, P.O. Box 1000, Tietotie 2, Espoo, 02044 VTT, Finland
e-mail: arja.laitila@vtt.fi

E. Kotaviita
Raisio plc, Research & Development, P.O. Box 101, 21201 Raisio, Finland

J. Olkku
Polttimo Companies Ltd, P.O. Box 22, 15141 Lahti, Finland

Introduction

Malting, the controlled germination of cereal grains, is a complex biological process involving a wide range of biochemical and physiological reactions. The main goal is the production of various enzymes capable of degrading the grain macromolecules into soluble compounds. Malting traditionally involves three stages: steeping, germination and kilning [2]. During the steeping stage, the moisture content of the grains is increased at 14–18°C up to 43–46% by alternating immersion and air rest periods. The grains are then allowed to germinate under humid and aerobic conditions at 16–20°C for 4–6 days. Finally, germination is terminated by kilning (drying) the grains for 24 h at temperatures increasing gradually from about 50 to 85°C or more depending on the type of malt. Kilning halts biochemical reactions and ensures microbiological stability of the dried product (moisture content

3–4%). Furthermore, several colour and flavour compounds are produced during kilning. Malt, traditionally made from barley (*Hordeum vulgare*), is a key material in beer production. In addition, malt is used for the production of distilled spirits, and can also be processed into ingredients for different branches of the food industry [2].

A diverse microbial community is associated with the barley grain, and consists of various types of bacteria, yeasts and filamentous fungi [25, 49, 52]. Therefore, malting can be considered as a complex ecosystem involving two metabolically active groups: the germinating grains and the diverse microbial community. Many intrinsic and extrinsic factors including plant variety, climate, soil type, agricultural practices, storage and transport influence the richness and structure of the microbial community present in the incoming barley [15, 25, 31, 52]. Furthermore, malting conditions are extremely favourable for microbial growth [25, 49]. Steeping can be considered as a critical step in malting with respect to microbiological safety. Although some of the microbes are washed away along with steep water draining, the viable number increases tremendously during the steeping period and remains high throughout the germination period [15, 24, 25, 52]. Finally, kilning reduces microbial activity. However, the number of microbes is generally higher in malt than in native barley [49]. The microbial community is also significantly influenced by malthouse operations, and each process step can be a source of additional microbes and their metabolites [24, 51, 52]. Thus, it is evident that the interactions between grains and microbes during malting greatly influence both malting performance and the properties of the final product [25, 49]. Depending on the nature and amount of microbes, these effects may be either beneficial or detrimental [6, 14, 18, 22, 25, 28, 32, 35, 36, 46, 49, 57, 61, 68, 72].

Although several studies of microbial communities in malting have been published, most of them have focused on bacteria and filamentous fungi. Relatively little has been reported on yeasts in industrial malting ecosystems. Flannigan [25] reported that yeasts are the second most abundant microbes after bacteria in viable counts in pre-harvest barley. Furthermore, encapsulated yeasts were reported to survive during long-term storage, whereas the number of bacteria and filamentous fungi associated with barley decreased [11]. High numbers of yeasts and yeast-like fungi have also been observed during the malting process [7, 15, 24, 25, 31, 51, 52]. Traditionally the yeasts in the malting ecosystem have been approximately divided into pink yeasts and a variety of white yeasts on the basis of colony morphology [15, 24, 25]. Previously, 10 ascomycetous and 6 basidiomycetous yeast species were reported from barley and from malting samples [15, 21, 23–25, 37, 49, 52, 68, 69]. Furthermore, a yeast-like fungus *Aureobasidium pullulans* was commonly encountered in pre- and post-harvest barley samples [11, 21, 24].

The role and the effects of yeasts in the malting ecosystem are not yet fully understood. Pigmented-yeasts may be responsible for discoloration of barley kernels and of grain products [37]. Yeasts also caused bridging of damp kernels during post-harvest storage in silos [48]. This extensive formation of yeast biomass around the kernels is also very likely to affect grain physiology during malting if uncontrolled yeast growth occurs during processing. Viable yeast cells of malt origin are destroyed at the latest by the high temperatures during mashing and wort boiling in the breweries [51], but it is well known that the microbial metabolites produced during malting may survive throughout the processing and enter the final product. Kreisz et al. [38] reported that small amounts of extracellular polysaccharides produced by malt-derived bacteria and yeasts may have a negative impact on wort and beer filtration. In addition, fungal activity on the malt husk appears to create factors that influence yeast flocculation, which is an important property of brewing yeast [70]. Furthermore, yeast metabolic activity in the production chain of fermented beverages may also lead to film formation, cloudiness and haziness, sediments and excessive gas production, off-odours and -flavours at all stages of the process [10, 26, 45].

Despite their several undesired characteristics, yeasts are extremely important microbes for the food and beverage industry. By production of valuable metabolites such as enzymes and vitamins, yeasts can contribute to the processability and nutritional value of cereal products [13, 63]. However, surprisingly little is known about their possible positive contribution to malt properties. Some yeasts normally associated with malting have shown strong antagonistic activity and have been applied as natural biocontrol agents to restrict the growth of harmful fungi [6, 17]. Our previous studies with lactic acid starter cultures revealed that the addition of lactic acid bacteria (LAB) into the steeping activated the indigenous yeast community and enhanced the production of microbial β-glucanase and xylanase in the malting process [32–30, 43]. However, the source of these microbial enzymes was unknown, and we suggested that enhanced growth of the yeast community could partly explain the increased enzyme activities. To our knowledge no research has been reported on the potential of yeasts from the industrial malting ecosystem to produce extracellular hydrolytic enzymes.

The aim of this study was to investigate the diversity of yeasts and yeast-like fungi in the industrial malting ecosystem. Furthermore, yeasts isolated from the malting process were screened for the production of extracellular hydrolytic enzymes. Better understanding of yeast ecology in the malting ecosystem could lead to more efficient control of the unwanted phenomena induced by yeasts as well as to the utilization of their beneficial properties in malt production.

Materials and methods

Sample collection

Four industrial lager malt production runs were monitored during the year 2002. In all cases the Finnish malting barley cultivar Saana from the 2001 crop was used. Barley was steeped twice in conical steeping vessels at about 15°C, with an 11–18-h air rest between the steeps. After steeping, the barley was transferred to germination boxes and germinated for 5–6 days at 15–20°C. The germination process was terminated by kilning. The air temperature was first raised to 55–60°C and finally to 85°C. The total kilning time was 16–19 h. A total of 136 samples were collected at nine stages; from the original barley ($n = 4$), from the steeping vessel after the air rest ($n = 28$) and after the steeping period ($n = 13$), from the germination boxes after 1 day germination ($n = 20$) and after the germination period prior to kilning ($n = 20$), from the dryer after 5 h kilning ($n = 18$), after 10 h kilning ($n = 16$), and after the whole kilning period ($n = 14$), and the final screened malt ($n = 3$). During steeping, 1–2 kg grain samples were taken through sampling pipes positioned at various locations and depths in the steeping vessels. A grain sampler was used to collect the samples from the various locations and depths in germination boxes and during kilning.

Enumeration and isolation

Samples (10 g) were mixed with 90 ml sterile saline solution, soaked at 4°C for 30 min and homogenized with a Stomacher Lab Blender 400 (Seward Medical, London, UK). Serial dilutions of homogenate were surface plated on yeast-malt extract agar, YM-agar (Difco Laboratories, Detroit, MI, USA), which was supplemented with 0.01% chlortetracycline (Sigma, St.Louis, MO, USA) and 0.01% chloramphenicol (Sigma) to prevent bacterial growth. In addition, 0.02% Triton-X 100 (BDH Laboratory Supplies, Poole, England) was used to prevent the spreading of fungal colonies. Replicate plates were cultivated in aerobic conditions at 15, 25 and 37°C for 5 days. Counts were expressed as colony forming units per gram (cfu/g).

A total of 733 colonies were isolated from the culture plates. Selection was based on different colony morphology (diameter, shape, colour and surface). Different colony types were collected throughout the malting process in order to identify the predominant species in the malting ecosystem. Cultures were purified by cross-streaking twice on YM agar, and stored in 10% glycerol at -70° and on YM-slants at 4°C for short-term storage.

DNA extraction

Genomic DNA for PCR reactions was extracted with two different methods. In the glass bead protocol the cells were grown on YM-agar at 25°C for 2 days. A loopful (10 μl) of cell mass was suspended in 1 ml redistilled water with 0.1 g 150–212 μm glass beads (Sigma-Aldrich, St Louis, MO, USA). Samples were homogenized in a FastPrep cell disrupter (FP120, Q-Biogene, Carlsbad, CA, USA) for 2 min at 5.5 m/s. Cell debris was removed by centrifugation at 13,000 rpm for 3 min. The supernatant containing the DNA was stored at −20°C.

The simple and rapid mechanical extraction protocol with glass beads was not efficient for most of the malting yeasts. Some yeast cells from the malting process were difficult to disrupt due to their very complex cell walls and capsules. Therefore, DNA was extracted from young cultures (18–24 h) with a DNA-kit, which combined both enzymatic treatment and mechanical lysis with ceramic sphere and garnet matrix. Genomic DNA from encapsulated yeasts was extracted with FastDNA®kit (Q-Biogene, Carlsbad, CA, USA) with CLS-Y lysing solution according to the manufacturer's instructions with minor modifications. A loopful (10 μl) of cell mass was suspended with 1 ml cell lysis solution (CLS-Y) in a tube containing lysing matrix (ceramic sphere and garnet matrix). Samples were homogenized in the FastPrep cell disrupter for 3 min at 5 m/s. Cell debris was removed by centrifugation at 12,000 rpm (14,000*g*) for 10 min. Purification and elution steps were carried out according to the manufacturer's instructions. DNA samples were stored at −20°C.

Molecular typing and identification

PCR-fingerprinting with M13 microsatellite primer (5′-GAGGGTGGCGGTTCT-3′) was performed according to Andrighetto et al. [1] with minor modifications.

Five microlitres of undiluted or 1:100 diluted DNA was mixed with 45 µl of PCR master mix. The master mix contained 1× DyNAzyme reaction buffer (final concentrations 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, pH 8.8), 200 µM of each dNTP, 2 U of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) and 1 µM of M13 primer (Sigma-Genosys, Cambridge, UK). Amplification was carried out in a UNO II Thermal Cycler (Biometra GmbH, Goettingen, Germany), with initial denaturation at 95°C for 5 min followed by 34 cycles of 60 s at 94°C, 20 s at 45°C (ramping to 72°C at 0.5°C−s^{-1}), 60 s at 72°C and 20 s at 50°C and a final extension at 72°C for 10 min. A reagent control in which DNA was replaced with redistilled water was included in every experiment. Amplified DNA fragments were separated by electrophoresis in 1.8% agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME, USA) in 0.5× TBE (Tris−Borate−EDTA, Bio-Rad laboratories, Hercules, CA, USA) buffer at 120 V for 4 h, and visualized by fluorescent labelling with ethidium bromide (Mercury, CLP, San Diego, CA, USA). Similarities between the DNA fingerprints of the selected isolates were determined with the aid of the Bionumerics program using the Unweighted Pairgroup Method with Arithmetic averages (UPGMA) clustering based on the Pearson correlation.

The strains were identified by sequence analysis of the D1/D2 domain of the 26S rRNA gene as described by Kurtzman and Robnett [40] except that the amplified DNA was purified with a QIAquick PCR purification kit (Qiagen, Mississaugua, Ontario, Canada), and the ABI BigDye v3.1 Terminator Cycle Sequencing kit was used for sequencing reactions (Applied Biosystems, Foster City, CA, USA). Electrophoresis of the products was carried out in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The sequences were edited with DNAMAN software version 4.1 (Lynnon Biosoft, Que, Canada). For identification, the sequences were compared with those of all known species available at the GenBank Database. A similarity of > 99% to 26S rRNA gene sequences was used as a criterion for identification [40]. Identified strains were deposited in the VTT Culture Collection and their nucleotide sequences were deposited in GenBank under the accession numbers shown in Tables 2 and 3.

Production of extracellular hydrolytic enzymes

The production of extracellular hydrolytic enzymes was studied with a plate-screening method. Yeast strains (27 ascomycetous and 28 basidiomycetous yeast) were cultivated on YNBG plates containing 0.67% yeast nitrogen base (YNB, Difco Laboratories), 1% glucose and 2% agar at 25°C for 2 days. Yeasts were then surface streaked on duplicate enzyme assay plates and incubated at 25°C for 3 days. Strains were screened for their ability to hydrolyse starch on a medium (YNBS) containing 0.67% YNB, 2% agar and 1% soluble starch (Merck, Darmstadt, Germany). After cultivation, the plates were stained with Lugol solution (containing iodine, 1 g; potassium iodine, 2 g and distilled water 300 ml). A yellow zone around a colony, in blue-stained medium, indicated amylase activity. β-Glucanase activity was determined on YNB plates (YNBB) supplemented with 0.5% barley β-glucan (P-BGBM, Megazyme, Wicklow, Ireland). Plates were flooded with 0.1% Congo red solution for 10–15 min and excess dye was rinsed off with 1 M NaCl. Clear halos around the colonies indicated hydrolysis of the substrate. Cellulase production was determined on YNBC plates containing 0.5% carboxymethylcellulose (CMC, Fluka, Buchs, Switzerland). For screening of hemicellulase activity, the YNB plates (YNBX) were supplemented with 1% xylan (Xylan from oat spelts, Sigma). The presence of extracellular cellulases and xylanase was detected with the Congo red method as described above.

Results and discussion

Yeast growth in the industrial malting ecosystem

The yeast community was monitored in four batches of Saana–barley from the 2001 crop as they went through the industrial malting processes from steeping to kilning (Table 1). The initial yeast count on stored barley was 2×10^4–2×10^5 cfu/g. The soaking of barley activated yeast growth, and a 10–100-fold increase of yeast counts was detected during the first days of malting. O'Sullivan et al. [51] reported an increase of the same magnitude in industrial scale, and Petters et al. [52] observed a 1,000-fold increase of yeasts during an industrial steeping period. Yeast activity remained high throughout the germination period, and the viable count reached a maximum level of 10^7 cfu/g at the end of germination.

We also studied the effect of growth temperature on the yeast counts. All the samples were incubated at 15, 25 and 37°C (Table 1). The majority of yeasts live in habitats in which the temperature domain is between 0 and 45°C [12]. As shown in Table 1, yeasts in the malting ecosystem were capable of growing at 15°C. Our results were in agreement with those of Petters et al.

Table 1 Yeast growth (cfu/g) during the industrial scale malting processes

Malting phase	*N*	Incubation temperature					
		15°C		25°C		37°C	
		Mean	Range	Mean	Range	Mean	Range
Barley	4	2×10^5	1×10^5–2×10^5	7×10^4	2×10^4–2×10^5	<50	<50
Steep, after air rest	28	4×10^5	1×10^5–7×10^5	3×10^5	3×10^4–1×10^6	1×10^3	<50–6×10^3
After steeping period	13	2×10^5	2×10^5–3×10^5	7×10^5	8×10^4–2×10^6	8×10^2	2×10^2–2×10^3
After 1 day germination	20	2×10^6	5×10^5–5×10^6	3×10^6	3×10^5–9×10^6	5×10^3	4×10^2–3×10^4
After germination	20	3×10^7	1×10^7–7×10^7	3×10^7	1×10^7–5×10^7	5×10^5	7×10^3–5×10^6
After 5 h kilning	18	4×10^7	2×10^7–6×10^7	3×10^7	1×10^7–6×10^7	6×10^5	4×10^3–4×10^6
After 10 h kilning	16	3×10^7	4×10^6–7×10^7	2×10^7	4×10^6–7×10^7	5×10^5	2×10^3–2×10^6
After kilning	14	2×10^6	9×10^5–3×10^6	2×10^6	7×10^5–4×10^6	3×10^4	<50–9×10^4
Screened malt	3	1×10^6	4×10^5–2×10^6	1×10^6	5×10^5–1×10^6	9×10^4	4×10^2–2×10^5

[52], who also found that the occurrence of yeasts in malting was attributed to their ability to grow at low temperatures.

Malting processes also harboured yeasts capable of growing at 37°C (Table 1). After the steeping periods 2×10^2–2×10^3 cfu/g were detected, and the viable count increased significantly during the germination and the initial phase of kilning. Thermotolerant yeasts represented ~2% of the yeast community after germination. Greater variation in the number of thermotolerant yeasts was observed within the batches compared to the populations growing at 15 or 25°C. Thermotolerant yeasts obviously originated from the malting equipment, as they were not detected in the native barley samples. Batch-to-batch variation in the process environment and in the malting procedures could explain the observed fluctuation. It has been shown that a specific microbial community develops in each malting plant [51, 52]. Furthermore, the yeast community present in the malting process depends on the initial population of barley, interactions between the different microbial groups, process conditions such as temperature and aeration, and the use of antimicrobial treatments such as starter cultures [25, 43, 49, 52].

Kilning appeared to have little effect on viable counts (Table 1). Kilning started with an air-on temperature of 55–60°C. Barley dried progressively from the bottom to the top of the grain bed, and the time that barley was exposed to each temperature depended on its location in the kiln. The first hours of kilning before the temperature breakthrough, especially in the top layers of the grain bed, appeared to favour yeast growth (data not shown). Only a tenfold reduction in yeast counts was observed during kilning. Yeast numbers in the finished malt ranged from 4×10^5 to 1×10^6 cfu/g. Schwarz et al. [60] also reported a large increase in ergosterol content during the early hours of kilning, indicating that the fungal growth was accelerated. Therefore, kilning can also be regarded as an important step with regard to the microbiological quality of malt. The kilning regime has been identified as a significant factor in controlling the microbial community [62]. Under normal environmental conditions, the vegetative yeast cells are rapidly inactivated by temperatures of 60–65°C [26]. However, in the malting ecosystem yeasts are well protected under the outer layers of the barley. Microbial populations adhere to external and internal surfaces of grain tissues to form a compact biofilm, which protects cells against heat and other antimicrobial treatments [66]. As discussed later, a large proportion of the yeast community was composed of encapsulating yeasts, which could also explain the high number of survivors in the kilned malt.

Characterization and identification of yeasts in the industrial malting ecosystem

The main goal of this study was to obtain an overall picture of the yeasts present in the industrial malting ecosystem. We isolated over 700 yeast colonies from various stages of the process. All the isolated yeasts were detected at least at a level of 10^4–10^5 cfu/g. Furthermore, the selection of different colony types from several samples throughout the malting process ensured that the predominant species were selected. However, some minor species may have been overlooked on the plates.

We applied DNA-based techniques for the differentiation of yeast isolates and for species identification. Yeast isolates were first discriminated with PCR-fingerprinting using an oligonucleotide primer (M13), targeting simple repetitive DNA sequences named microsatellites. This protocol has been widely applied in yeast typing and it allows discrimination of species even at the subspecies level [44]. In this study 55 fingerprint patterns were detected. All the yeast isolates with different DNA-fingerprints were further identified with sequencing of

the DNA sequence (600–650 nucleotides) from the 5′ end of the 26S rRNA gene, D1/D2 region [19, 40].

A surprisingly diverse yeast community was detected in the industrial malting processes of Saana-01 barley. We detected 25 species of ascomycetous yeasts belonging to eight different genera (Table 2) and 18 species of basidiomycetous yeasts belonging to six different genera (Table 3). The most frequently encountered ascomycetous genera were *Candida*, *Clavispora, Galactomyces, Hanseniaspora, Issatchenkia, Pichia, Saccharomyces* and *Williopsis* (Table 2). In addition, two ascomycetous yeast-like fungi or black yeasts, *A. pullulans* and *Exophiala dermatidis*, were frequently detected. *Candida* species were allocated to 11 different species, namely *C. anglica*, *C. cylindracea*, *C. fermentati*, *C. intermedia*, *C. natalensis*, *C. pararugosa*, *C. picinguabensis*, *C. saitoana*, *C. sake*, *C. silvae* and *C. solani*. Furthermore, two sets of isolates *Candida* sp. I (C-04530) and II (C-04532) did not match closely enough to any of the sequences present at the time in the database query. *Candida* sp. I gave only 92.8% similarity to an undescribed *Candida* sp. and strain IV 93.9% to *Issatchenkia* sp. These two isolates may represent novel *Candida* or *Issatchenkia* species and are being subjected to further characterization.

Basidiomycetous yeasts comprised six different genera: *Bulleromyces, Cryptococcus, Filobasidium, Rhodotorula, Sporobolomyces* and *Trichosporon* (Table 3). A wide variety of white, cream and pigmented *Cryptococcus* species were detected in the malting ecosystem. They were identified as *C. albidosimilis, C. curvatus, C. hungaricus, C. macerans, C. magnus, C. victoriae* and *C. wieringae*. In addition, four groups of undescribed *Cryptococcus* species, indicated as *Cryptococcus* sp. I–IV, were found on the basis of D1/D2 sequences. *Cryptococcus* sp I (VTT C-04545) showed 99.4%

Table 2 Ascomycetous yeasts identified by sequence analysis of the 26S rRNA D1/D2 region

Genera	Species	VTT Culture collection number	Sequence similarity to the closest species/strain		GeneBank Account number
			%	bp	
Candida	*C. anglica*[a], *smooth colony type*	C-04516	100	570/570	DQ377631
	C. anglica[a], *rough colony type*	C-04517	100	570/570	DQ377632
	C. cylindracea	C-04529	99.1	568/573	DQ377633
	C. fermentati	C-04519	100	534/534	DQ377634
	C. intermedia	C-04520	99.8	518/519	DQ377635
	C. natalensis	C-04521	100	569/569	DQ377636
	C. pararugosa	C-04522	100	581/581	DQ377637
	C. picinguabensis	C-04523	100	488/488	DQ377638
	C. saitoana	C-04524	100	538/539	DQ377639
	C. sake	C-04518	100	587/587	DQ377640
	C. silvae	C-04527	100	541/541	DQ377641
	C. solani	C-04528	100	568/568	DQ377642
	Candida sp. II	C-04530	92.8	323/348	DQ377643
	Candida sp. IV	C-04532	93.9	526/560	DQ377644
Clavispora	*Cl. lusitaniae*	C-04533	99.8	551/552	DQ377645
Galactomyces	*G. geotrichum*[b]		nd	nd	
	Geotrichum silvicola	C-04559	99.6	559/561	DQ377646
Hanseniaspora	*H. clermontiae/meyri*	C-04560	99.8[c]	583/584	DQ377647
	H. uvarum	C-04561	100	556/556	DQ377648
Issatchenkia	*I. orientalis*	C-04562	100	602/602	DQ377649
Pichia	*P. anomala*	C-04565	100	573/573	DQ377650
	P. fabianii	C-04566	100	613/613	DQ377651
	P. fermentans	C-04567	100	556/556	DQ377652
	P. guilliermondii	C-04568	100	561/561	DQ377653
Saccharomyces	*S. exiguous*	C-04572	99.5	586/589	DQ377654
Williopsis	*W. californica*	C-04576	100	632/632	DQ377655
Black yeasts					
Aureobasidium	*A. pullulans*	D-041013	100	614/614	DQ377656
Exophiala	*E. dermatidis*	D-041016	100	617/617	DQ377657

[a] Two different colony types of *C. anglica* were deposited in the VTT culture collection although they gave similar DNA-fingerprinting patterns and identical sequences

[b] *G. geotrichum* was identified on the basis of morphology and DNA-fingerprints. Pure cultures, previously isolated and identified from the industrial malting processes (VTT C-94425 and VTT C-99718), were compared to those isolated in this study

[c] Could not be separated by D1/D2 sequencing

Table 3 Basidiomycetous yeasts identified by sequence analysis of the 26S rRNA D1/D2 region, VTT Culture Collection number and GeneBank access number

Genera	Species	VTT Culture collection number	Sequence similarity to the closest species/strain		GeneBank Account number
			%	bp	
Bulleromyces	*B. albus*	C-04514	100	632/632	DQ377658
Cryptococcus	*C. albidosimilis*	C-04508	99.8	611/612	DQ377659
	C. curvatus	C-04536	99.9	507/508	DQ377660
	C. hungaricus	C-04558	98.8	619/629	DQ377661
	C. macerans	C-04538	99.8	624/625	DQ377662
	C. magnus	C-04540	99.8	643/644	DQ377663
	C. victoriae	C-04542	100	489/489	DQ377664
	C. wieringae	C-04509	100	626/626	DQ377665
	Cryptococcus sp.I	C-04545	99.4	618/622	DQ377666
	Cryptococcus sp.II[a]	C-04546	99.8	602/603	DQ377667
		C-04547	100	603/603	DQ377668
	Cryptococcus sp.III[a]	C-04548	99.8	628/629	DQ377669
		C-04549	99.8	639/640	DQ377670
		C-04550	99.8	634/635	DQ377671
		C-04551	99.4	637/641	DQ377672
	Cryptococcus sp.IV[a]	C-04510	100	532/532	DQ377673
		C-04552	100	611/611	DQ377674
		C-04553	100	611/611	DQ377675
		C-04554	100	611/611	DQ377676
		C-04555	100	611/611	DQ377677
		C-04556	100	611/611	DQ377678
		C-04557	100	611/611	DQ377679
Filobasidium	*F. globisporum*	C-04511	99.4	627/631	DQ377680
Rhodotorula	*R. glutinis*	C-04513	100	617/617	DQ377681
	R. pinicola	C-04570	100	626/627	DQ377682
Sporobolomyces	*S. roseus*	C-04574	100	604/604	DQ377683
	S. ruberrimus	C-04573	100	597/597	DQ377684
Trichosporon	*T. brassicae*	C-04575	100	626/626	DQ377685

[a] Members of the *Cryptococcus* II–IV groups gave several banding patterns with microsatellite primers and strains with distinct fingerprints have been deposited to VTT culture collection

similarity to *Cryptococcus* strain CBS 7743 (Gene bank no. AJ311452), which was closely related to *C. nyarrowii*. These strains were previously isolated from soil and snow from Antarctica [67]. The D1/D2 sequences of the *Cryptococcus* sp. II isolates were identical with those of *Cryptococcus* yeasts isolated from soil in Austria (HB946, Genbank no. AJ510201), and for the isolates of the type III the closest relative was another *Cryptococcus* HB1052 strain isolated from soil in Austria (Genbank no AJ510146). The sequence of the *Cryptococcus* sp. IV was identical with that of the strain KCTC 17065 (Genbank no. AF459681) isolated from flower samples in Korea [33]. *Cryptococcus* isolates I–IV have been subjected to further phenotypic and genotypic characterization.

Occurrence of yeasts in the various stages of the malting process

The species detected in the various stages of the malting process are summarized in Table 4. Basidiomycetous yeasts dominated in the yeast community of barley. In addition they were frequently detected during the first days of malting. The growth of basidiomycota was favoured by low temperatures during steeping. Many species have temperature optima below 20°C [12, 67]. The oxidative basidiomycetous yeasts are common in plant ecosystems [25, 27]. The attachment of these yeasts to plant surfaces is attributed to the production of extracellular gums and mucilages, which also protects the cells from desiccation and other external factors [8]. This study clearly shows that encapsulated basidiomycetous yeasts can survive the high temperatures reached during kilning. Basidiomycetous yeasts also have other survival properties, such as the ability to compete with other organisms for nutrients, which could their abundance in the barley ecosystem [27]. In addition, *Cryptococcus, Rhodotorula* and *Sporobolomyces* generally produce red, pink and yellow carotenoid pigments, which enhance tolerance to sunlight and radiation [27]. Yeast pigments may later be involved in the discoloration of grains and cereal products.

Table 4 The predominant yeast species in the various stages of malting

Ascomycetous yeasts							
A. pullulans	*C. anglica* *C. fermentati*[a] *C. intermedia*[a] *C. natalensis* *C. pararugosa*[a] *C. silvae* *P. fabianii*[a] *P. fermentans*[a] *A. pullulans*	*C. anglica* *C. fermentat*[a] *C. pararugosa* *Candida sp. I*[a] *G. geotrichum* *P. anomala* *P. fermentans*[a] *P. guilliermondii*[a] *W. californica* *A. pullulans* *E. dermatidis*	*C. anglica* *C. natalensis* *C. pararugosa*[a] *C. solani* *Cl. lusitaniae*[a] *G. geotrichum* *P. guilliermondii* *W. californica* *A. pullulans* *E. dermatidis*	*C. anglica* *C. fermentati*[a] *C. natalensis* *C. pararugosa*[a] *C. saitoana* *C. silvae* *Cl. lusitaniae*[a] *G. geotrichum* *H. clermontiae* *I. orientalis*[a] *P. anomala* *P. fabianii*[a] *P. fermentans*[a] *P. guilliermondii*[a] *W. californica*	*C. anglica* *C. cylindracea* *C. natalensis* *C. pararugosa*[a] *C. picinguabensis*[a] *C. saitoana* *C. silvae* *C. solani* *Cl. lusitaniae*[a] *G. geotrichum* *H. clermontiae* *H. uvarum* *I. orientalis*[a] *P. anomala* *P. fabianii*[a] *P. fermentans*[a] *P. guilliermondii*[a] *S. exiguus* *A. pullulans* *E. dermatidis*	*C. anglica* *C. cylindracea* *C. pararugosa* *C. sake* *C. saitoana* *C. silvae* *Cl. lusitaniae*[a] *H. uvarum* *P. anomala* *P. fabianii*[a] *P. fermentans*[a] *P. guilliermondii*[a] *A. pullulans*	*C. anglica* *C.natalensis* *C. pararugosa*[a] *C. saitoana* *C. silvae* *Candida sp. II* *Cl.lusitaniae*[a] *G. geotrichum* *G. silvicola* *H. uvarum* *I. orientalis*[a] *P. fabianii*[a] *P. fermentans*[a] *P. guilliermondii*[a] *A. pullulans*
Barley	Steeping		Germination		Kilning		
	After air rest	After steeping	After 1 day germination	After germination	After 5 h kilning	After 10 h kilning	Malt isolates
isolates (n = 20)	Isolates (n = 113)	Isolates (n = 104)	Isolates (n = 119)	Isolates (n = 79)	Isolates (n = 101)	Isolates (n = 84)	(n = 113)
Basidiomycetous yeasts							
B. albus *C. albidosimilis* *C. magnus* *C. wieringae* *Cryptococcus* sp IV *F. globisporum* *R. glutinis* *S. ruberrimus*	*B. albus* *C. albidosimilis* *C. hungaricus* *C. macerans* *C. victoriae* *C. wieringae* *Cryptococcus sp I* *Cryptococcus* sp IV *F. globisporum* *R. glutinis* *S. ruberrimus* *T. brassicae*[a]	*B. albus* *C. hungaricus* *C. victoriae* *C. wieringae* *Cryptococcus* sp II *Cryptococcus* sp III *Cryptococcus* sp IV *F. globisporum* *R. glutinis* *S. ruberrimus*	*B. albus* *C. albidosimilis* *C. curvatus*[a] *C. victoriae* *C. wieringae* *Cryptococcus* sp II *Cryptococcus* sp III *Cryptococcus* sp IV *F. globisporum* *R. glutinis* *S. ruberrimus*	*Cryptococcus* sp III *Cryptococcus* sp IV *R. glutinis* *S. roseus* *S. ruberrimus*	*Cryptococcus* sp III *Cryptococcus* sp IV *R. glutinis* *R. pinicola* *S. ruberrimus*	*C. magnus* *Cryptococcus* sp III *Cryptococcus* sp IV *R. glutinis* *R pinicola* *S. ruberrimus*	*B. albus* *C. macerans* *C. magnus* *Cryptococcus* sp IV *R. glutinis* *S. ruberrimus*

[a] Species detected after 37°C cultivation

The following six basidiomycetous yeasts: *C. albidus, Cryptococcus* sp. *R. glutinis, R. mucilaginosa, S. roseus* and *T. beigelii* have previously been reported in the malting process [15, 21, 23, 25, 49, 52, 69]. We found 11 different *Cryptococcus* species in the malting process including four potentially novel species. *Cryptococcus* species are commonly found in both natural and man-made ecosystems. *Cryptococcus* species as well as *Filobasidium globisporum* were encountered especially during steeping and at the beginning of germination. *F. globisporum* is also known from plant material and is often found in weathered leaves [4, 39]. This study showed that *Bulleromyces albus* (anamorph *Bullera alba*), commonly associated with plant ecosystems [4], also survived throughout the malting process.

In agreement with previous investigations [24, 25, 49, 52, 69], the red-pigmented yeasts *Rhodotorula* and *Sporobolomyces* were important members of the malting ecosystem. *Rhodotorula* species can be found from a variety of substrates and environments world-wide [39]. *Sporobolomyces* species mainly occur in the phyllosphere [5, 20]. *R. glutinis* was isolated in every malting sample (Table 4). In addition we detected *R. pinicola* in samples from the initial phase of kilning. This species was recently isolated from pine twigs [74]. We detected the presence of two *Sporobolomyces* species during malting. *S. ruberrimus* was isolated at every stage and *S. roseus* at the end of germination. We also isolated one member of the *Trichosporon* genus during steeping; *T. brassicae*, which has previously been detected from cabbage [39].

In contrast to basidiomycetous yeasts, ascomycetous species dominated at the end of germination and especially during the first hours of kilning. We found 20 different ascomycetous yeasts in the samples taken after 5 h of kilning, whereas only five basidiomycetous species were detected in the same samples (Table 4). The occurrence of ascomycetous yeasts was obviously due to their ability to grow better at the higher temperatures prevailing at the end of malting than basidiomycetous yeasts. The predominant yeast species detected in 37°C cultivations were *C. fermentati, C. intermedia, C. pararugosa, Candida* sp I, *Cl. lusitaniae, I. orientalis, P. fabianii, P. fermentans* and *P. guilliermondii*. Only two basidiomycetous yeasts, *C. curvatus* and *T. brassicae*, were detected in YM-plates cultivated at 37°C.

Candida species were among the most frequently detected ascomycota throughout the malting process. This study revealed 13 different species in the malting ecosystem. Only three different species have previously been reported from malting processes [25, 49, 52]. *C. anglica, C. pararugosa*, *C. natalensis* and *C. silvae* were present in every malting stage including the final malt. The genus *Candida* is an extremely heterogenous group of yeasts and is commonly associated with plants, rotting vegetation, insects which feed on plants, or with different foods and beverages [39, 41, 54, 58].

This study showed that *Clavispora lusitaniae* was part of the malting ecosystem and was detected in samples derived from germination and kilning. It was frequently isolated from 37°C cultivations. *C. lusitaniae* occurs in a wide variety of substrates of plant and animal origin as well as in industrial wastes and clinical specimens [39, 42].

Galactomyces geotrichum (anamorph *Geotrichum candidum*) was a common isolate in process samples. In addition to *G. geotrichum* another *Geotrichum* species, *G. silvicola*, was isolated from the malt samples (Table 4). *G. geotrichum* is generally considered as a process contaminant and is rarely present in native barley, but may become one of the dominant fungi during germination and is often found in kilned products [52]. Douglas and Flannigan [15] reported that increased aeration during steeping may promote the growth of *Geotrichum*. They also reported that contamination with *G. geotrichum* led to reduction of the other yeast-like fungi. Subsequently, *G. geotrichum* with antifungal properties has found an application in the malting industry as a biocontrol agent for prevention of toxigenic fungi [6].

Members of the genus *Hanseniospora* became part the yeast community at the end of germination and were detected particularly during the initial phase of kilning. We detected two different species, *H. clermontiae* and *H. uvarum*, which has previously been reported in the malting process [52]. Furthermore, species of *Hanseniaspora* are common organisms in soil, various plants and fruits and are particularly associated with grapes and wine production [27, 53].

Issatchenkia orientalis was isolated from the samples derived from germination and kilning. It also entered the final product. *I. orientalis* is also commonly associated with various foods such as fruit juice, tea, beer, bread, dairy products, fermented foods and beverages [39]. It has often been linked with food and beverage spoilage and particularly with film formation [53].

The *Pichia* population during malting was composed of four species: *P. anomala*, *P. fabianii, P. fermentans* and *P. guillliermondii*. All of them were detected in every stage of the process except for *P. anomala*, which was isolated only during the first hours of kilning. *Pichia* are also an extremely heterogenous group of yeasts [39]. They are common in both natural and clinical environments and are often found in industrial fermentation processes [3, 39]. *P. guilliermondii* is often isolated from insects such as beetles, which are the

principal vectors for the transportation of yeasts in plant ecosystems [65]. *P. anomala* and *P. guilliermondii* strains with antagonistic activity have been applied as biocontrol agents to suppress pre- and post-harvest fungal diseases [16, 55].

Only one representative of the genus *Saccharomyces*, *S. exiguus* was detected during the first hours of kilning. This species is often associated with fresh and processed vegetables as well as with spoilage of soft drinks and fruit juices [39]. It is also a common organism in sour doughs [12]. *Williopsis californica* was mainly found in the samples derived from steeping and germination. This species is widely distributed in nature, commonly being found in soil, streams, lakes and plants [39].

Ascomycetous yeast-like fungi were frequently encountered in the malting ecosystem. *A. pullulans* was already present in the indigenous microbial community of barley, and was also detected in every process step (Table 4). Several studies have shown that *A. pullulans* is a very common organism in pre- and post-harvest barley samples as well as in the malting process [15, 21, 22, 24, 52]. *A. pullulans* is a ubiquitous saprophyte in the phyllosphere and is often found in decaying and damp materials [53, 73]. It has been reported from various foods, but only rarely linked to food spoilage [53]. It is also a potential biocontrol agent of plant pathogens [59].

The other black yeast, *E. dermatidis*, was detected in samples derived from steeping, germination and kilning, but not in the native barley. Thus, it can be considered as a process contaminant. *E. dermatidis* has a world-wide distribution and has been isolated especially from environments with high temperature, high moisture and low nutrient levels such as bathrooms, saunas and steam rooms [47]. The cells are protected by extracellular polysaccharides, which promote their survival in harsh environments. These properties could also explain their frequent occurrence in the malting environment. Although this organism is commonly found in various man-made environments, its natural ecological niche is unknown [47].

It is evident that a great number of different yeast species play a significant role in the industrial malting ecosystem, and that yeast association with grains may be more important than previously believed. In this study nine species were predominant in native barley and 21 different species formed the prevalent yeast community in the screened malt. Although only one cultivar from one crop year was followed throughout the processing, this study provides a clear indication of the vast microbial diversity in commercial scale malting. It is obvious that even more heterogeneity could be expected due to differences between crops as well as between industrial practices in different locations.

Production of extracellular hydrolytic enzymes

Malted grains can be considered as packages of enzymes and nutrients utilized especially in beer production. The successful production of malt includes production of various hydrolytic enzymes and controlled degradation of the grain endosperm structure. The key component of barley of interest to the brewer is the starchy endosperm, which represents about 70% of the total weight [2]. The cell walls of barley contain 70% [1, 3], (1,4)-β-D-glucan, 20% arabinoxylan, 6% protein, 2% cellulose and small amounts of other components [71]. It is now recognised that the microbial community associated with the grains has a significant impact on malt enzyme potential. Several studies have indicated that filamentous fungi present in barley and in malting produce a wide range of enzymes [22, 23, 35, 50, 56, 61, 72]. Although yeasts are important players in the malting ecosystem, their contribution to malting and brewing performance is still not fully understood. In this study, yeasts and yeast-like fungi isolated from industrial malting processes were screened for the production of amylase, β-glucanase, cellulase and xylanase. To our knowledge this is the first report on enzyme profiles of yeasts isolated from the malting environment.

The enzyme profiles were determined using minimal medium containing 0.5 -1% of substrate as sole carbon source (Tables 5 and 6). All the yeast grew in YNB supplemented with glucose, which was used as a positive control. Ascomycetous yeasts in general were not able to utilise complex polysaccharides as the only source of energy in the plate-screening assay. An exception was the yeast-like fungus *A. pullulans*, which was an effective degrader of all the substrates tested. In addition, *E. dermatidis* hydrolysed both barley β-glucan and carboxymethylcellulose. We also found that *G. silvicola* (C-04559) degraded cellulose. As shown in Table 6, basidiomycetous yeasts were active in the production of glucanase, cellulase and xylanase. Several basidiomycetous yeasts also hydrolysed starch. The most intensive degradation was observed with *C. macerans*, *S. roseus* and *S. ruberrimus* isolates. In addition, several species exhibited β-glucanase activity. The most significant producers of β-glucanase were *B. albus, C. macerans, C. magnus*, *F. globisporum* and *R. pinicola.* All these species, except *R. pinicola*, also showed cellulase activity. Cellulose and xylan degradation was detected especially among the *Cryptococcus* species. Many of these activities are assumed to be

Table 5 Production of extracellular enzymes by ascomycetous yeasts isolated from an industrial malting ecosystem

Genera	Species	VTT number	Amylase	β-Glucanase	Cellulase	Xylanase
Candida	*C. anglica*	C-04516	–	–	–	–
	C. anglica	C-04517	–	–	–	–
	C. cylindracea	C-04529	–	–	–	–
	C. fermentati	C-04519	–	–	–	–
	C. intermedia	C-04520	–	–	–	–
	C. natalensis	C-04521	±	–	–	–
	C. pararugosa	C-04522	–	–	–	–
	C. picinguabensis	C-04523	–	–	–	–
	C. saitoana	C-04524	–	–	–	–
	C. sake	C-04518	–	–	–	–
	C. silvae	C-04527	–	–	–	–
	C. solani	C-04528	–	–	–	–
	Candida sp I	C-04530	–	–	–	–
	Candida sp II	C-04532	–	–	–	–
Clavispora	*C. lusitaniae*	C-04533	–	–	–	–
Geotrichum	*G. silvicola*	C-04559	–	–	+++	–
Hanseniaspora	*H. clermontiae.*	C-04560	–	–	–	–
	H. uvarum	C-04561	–	–	–	–
Issatchenkia	*I. orientalis*	C-04562	–	–	–	–
Pichia	*P. anomala*	C-04565	–	–	–	–
	P. fabianii	C-04566	–	–	–	–
	P. fermentans	C-04567	–	–	–	–
	P. guilliermondii	C-04568	–	–	–	–
Saccharomyces	*S. exiguus*	C-04572	–	–	–	–
Williopsis	*W. californica*	*C-04576*	–	–	–	–
Black yeasts						
Aureobasidium	*A. pullulans*	D-041013	+++	+++	+++	++
Exophiala	*E. dermatidis*	D-041016	–	+++	++	–

+...++ positive result: a clear zone, +++ intensive degradation of the substrate, – negative result: no degradation, ± weak production

necessary for the fungi to degrade barley cell wall components and to penetrate plant cell walls [22].

In agreement with earlier research [9, 64], this study shows that yeasts are potential producers of enzymes degrading plant cell walls. However, some enzyme activities, especially those of ascomycetous yeasts, may have been overlooked in the plate screening method in which the complex carbohydrate is the only source for energy. Strauss et al. [64] reported that some ascomycetous yeasts showed cellulase activity only in the presence of glucose. Horn [34] reported that growth of the ascomycetous yeast *Pichia guilliermondii* associated with corn was considerably increased in association with amylolytic filamentous fungi. In the malting ecosystem complex interactions with the other microbial groups such as filamentous fungi and bacteria as well as with the germinating grain influence the growth and activity of the yeast community. Yeasts may also be a source of proteolytic and lipolytic activities. Although these enzymes have not been as extensively studied as polysaccharide hydrolysing enzymes, they are known to influence malt quality. Our further work will include studies on the microbial contribution to these enzymes.

Conclusions

This study revealed that the indigenous yeast community in the industrial malting ecosystem is complex and consists of a wide variety of ascomycetous and basidiomycetous yeasts. Although some minor species may have been overlooked in this study, the diversity of the yeast community in barley malting is greater than expected. Some potentially novel species were found in the malting ecosystem. Many of the yeasts associated with barley and malting produced enzymes degrading plant cell walls, which may contribute to the enzyme potential of malt, although their significance in industrial malt production needs to be confirmed. Knowledge of the microbial ecology of the malting process provides a basis for microflora management and understanding of the role of microbes in the malting ecosystem.

Acknowledgements The authors are grateful to the staff of malthouses for help with the industrial trials. We acknowledge the technical staff of VTT for their skilful assistance, especially Tarja Nordenstedt and Merja Salmijärvi for microbiological assistance and Anne Heikkinen for assistance with the preparation of culture media. We thank Michael Bailey for critical reading of the

Table 6 Production of extracellular enzymes by basidiomycetous yeasts isolated from an industrial malting ecosystem

Genera	Species	VTT number	Amylase	β-Glucanase	Cellulase	Xylanase
Bulleromyces	*B. albus*	C-04507	+/−	+++	+++	+
Cryptococcus	*C. albidosimilis*	C-04508	+/−	+/−	−	+
	C. curvatus	C-04536	−	+	−	+
	C. hungaricus	C-04558	++	−	−	−
	C. macerans	C-04538	+++	+++	+++	−
	C. magnus	C-04540	−	++	+++	+
	C. victoriae	C-04542	−	−	−	+
	C. wieringae	C-04509	+	−	+++	+
	Cryptococcus sp.I	C-04545	+	−	+++	−
	II	C-04546	+	−	+++	+/−
		C-04547	+	−	+++	+/−
	III	C-04548	−	−	+++	+
		C-04549	−	−	+++	+
		C-04550	−	+/−	+++	+
		C-04551	−	−	−	−
	IV	C-04510	−	−	−	+
		C-04552	−	−	−	+
		C-04553	−	+	−	+
		C-04554	−	−	−	+
		C-04555	−	−	−	+
		C-04556	−	−	−	+
		C-04557	−	−	−	+
Filobasidium	*F. globisporum*	C-04511	−	+++	+++	−
Rhodotorula	*R. glutinis*	C-04513	−	−	−	−
	R. pinicola	C-04570	−	++	−	−
Sporobolomyces	*S. roseus*	C-04574	+++	−	−	−
	S. ruberrimus	C-04573	+++	−	−	−
Trichosporon	*T. brassicae*	C-04575	−	−	−	−

+... + + positive result: a clear zone, + + + intensive degradation of the substrate, − negative result: no degradation, + /− weak production

manuscript. This research was supported by the Finnish Malting and Brewing Industry, by the TEKES, the National Technology Agency of Finland, and by the Raisio Group Research Foundation.

References

1. Andrighetto G, Psomas E, Tzanetakis N, Suzzi G, Lomardi A (2000) Randomly amplified polymorphic DNA (RAPD) PCR for the identification of yeasts isolated from dairy products. Lett Appl Microbiol 30:5–6
2. Bamforth C, Barclay A (1993) Malting technology and the uses of malt. In: Barley: Chemistry, Technology; MacGregor A, Bhatty R (eds) American Association of Cereal Chemists Inc, St Paul, MN, pp 297–354
3. Barnett J, Payne R, Yarrow D (2000) Yeasts: characterization and identification, 3rd edn. Campridge University Press, Cambridge, UK
4. Boekhout T, Phaff H (2003) Yeast biodiversity. In: Boekhout T, Robert V (eds) Yeast in food. Beneficial and Detrimental Aspects. Behr's Verlag GmbH & Co., Hamburg, pp 1–38
5. Boekhout, Nakase (1998) Sporobolomyces kluyver and van Niel. In: Kurzamn C, Fell J (eds) The yeasts, a taxonomic study, 4th edn. Elsevier Science BV, Amsterdam, The Netherlands, pp 731–741
6. Boivin P, Malanda M (1997) Improvement of malt quality and safety by adding starter culture during the malting process. Tech Q Master Brew Assoc Am 34:96–101
7. Bol J, Huis in't Veld J (1988) The occurrence of fungi on barley, in raw materials and in the malthouse: prevention and consequences of infection. Louv Brew Lett 1:4–9
8. Bose I, Reese A, Ory J, Janbon G, Doering T (2003) A yeast under cover: the capsule of *Cryptococcus neoformans*. Eukaryot Cell 2:655–663
9. Buzzini P, Martini A (2002) Extracellular enzymatic activity profiles in yeast and yeast-like strains isolated from tropical environments. J Appl Microbiol 93:1020–1025
10. Campbell I (2003) Wild yeasts in brewing and distilling. In: Priest FG, Campell I (eds) Brewing Microbiology, 2nd edn. Chapman & Hall, London, UK, pp 247–266
11. Clarke J, Hill S (1981) Mycofloras of moist barley during sealed storage in farm and laboratory silos. Trans Br Mycol Soc 77:557–565
12. Deak T (1991) Foodborne yeasts. Adv Appl Microbiol 36:179–278
13. Demain A, Phaff H, Kurtzman C (1998) The industrial and agricultural significance of yeasts. In: Kurtzman C, Fell J (eds) The yeasts, a taxonomic study. Elsevier Science BV, Amsterdam, The Netherlands, pp 13–30
14. Doran P, Briggs D (1993) Microbes and grain germination. J Inst Brew 99:165–170
15. Douglas P, Flannigan B (1988) Microbiological evaluation of barley malt production. J Inst Brew 94:85–88
16. Druvefors U, Jonsson N, Boysen M, Schnürer J (2002) Efficacy of the biocontrol yeast *Pichia anomala* during long-term storage of moist feed grain under different oxygen and carbon dioxide regimens. FEMS Yeast Res 2:389–394

17. Dziuba E, Foszcynska B (2001) Biological protection of barley grain and its influence on selected features of malt. Pol J Food Nutr Sci 10(51):49–53
18. Etchevers G, Banasik O, Watson C (1977) Microflora of barley and its effect on malt and beer properties: a review. Brew Dig 52:46–53
19. Fell J, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A (2000) Biodiveristy and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int J Syst Evol Microbiol 50:1351–1371
20. Fell J, Scorzetti G, Statzell-Tallman A, Pinel N, Yarrow D (2002) Recognition of the basidiomycetous yeast *Sporobolomyces ruberrimus* sp. nov. as a distinct species based on molecular and morphological analyses. FEMS Yeast Res 1:265–270
21. Flannigan B (1969) Microflora of dried barley grain. Trans Br Mycol Soc 53:371–379
22. Flannigan B (1970) Degradation of arabinoxylan and carboxymethyl cellulose by fungi isolated from barley kernels. Trans Br Mycol Soc 55:277–281
23. Flannigan B, Dickie N (1972) Distribution of microorganisms in fractions produced during pearling of barley. Trans Br Mycol Soc 59:377–391
24. Flannigan B, Okagbue R, Khalid R, Teoh C (1982) Mould flora of malt in production and storage. Brew Distill Int 12:31–33, 37
25. Flannigan B (1996) The microflora of barley and malt. In: Priest FG, Campell I (eds) Brewing microbiology, 2nd edn. Chapman & Hall, London, UK, pp 83–125
26. Fleet G (1992) Spoilage yeasts. Crit Rev Biotechnol 12:1–44
27. Fleet G (2003) Yeasts in fruit and fruit products. In: Boekhout T, Robert V (eds) Yeast in food. Beneficial and Detrimental Aspects. Behr's Verlag GmbH & Co, Hamburg, pp 267–287
28. Haikara A, Home S (1991) Mash filtration difficulties caused by the split barley kernels: a microbiological problem. In: Proceeding of the European brewing conversation, Lisbon, Portugal, pp 537–544
29. Haikara A, Laitila A (1995) Influence of lactic acid starter cultures on the quality of malt and beer. In: Proceeding of the European brewing conversation, Brussels, Belgium, pp 249–256
30. Haikara A, Laitila A (2001) The use of lactic acid bacteria cultures in malting improvement. In: Andrews M, Andrews ME, Humphry D (eds) Plant microbial interactions: positive interactions in relation to crop production and utilisation. The Association of Applied Biologist, Warwick, UK, pp 229–238
31. Haikara A, Mäkinen V, Hakulinen R (1977) On the microflora of barley after harvesting, during storage and in malting. In: Proceeding of the European brewing conversation, Amsterdam, The Netherlands, pp 35–46
32. Haikara A, Uljas H, Suurnäkki A (1993) Lactic starter cultures in malting–a novel solution to gushing problems. In: Proceeding of the European brewing conversation, Oslo, Norway, pp 163–172
33. Hong S, Lee K, Bae K (2002) Diversity of yeasts associated with natural environments in Korea. J Microbiol 40:55–62
34. Horn B (1984) Association of *Candida guilliermondii* with amylolytic filamentous fungi on preharvest corn. Can J Microbiol 31:19–23
35. Hoy J, MacCauley B, Fincher G (1981) Cellulases of plant and microbial origin in germinating barley. J Inst Brew 87:77–80
36. Kelly L, Briggs D (1992) The influence of the grain microflora on the germinative physiology of barley. J Inst Brew 98:395–400
37. Kottheimer J, Christensen C (1961) Microflora of barley kernels. Wallerstein Lab Commun 24:21–27
38. Kreisz S, Wagner F, Back W (2001) The influence of polysaccharides from yeast and bacteria on the filterability of wort and beer. In: Proceeding of the European Brewing Conversation, Budapest, pp 1–9
39. Kurtzman C, Fell J (1998) The Yeasts, a taxonomic study. Elsevier Science BV, Amsterdam, The Netherlands
40. Kurtzman C, Robnett C (1998) Identification and phylogeny of ascomycetous yeasts for analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek 73:331–371
41. Kurztman C, Robnett C, Yarrow D (2001) Three new species of *Candida* from apple cider: *C. anglica*, *C. cidri* and *C. pomola*. Ant Leeuw 80:237–244
42. Lachance M, Daniel H, Meyer W, Prasad G, Gautam S, Boundy-Mills K (2003) The D1/D2 domain of the large-subunit rDNA of the yeast species *Clavispora lusitaniae* is unusually polymorphic. FEMS Yeast Res 4:253–258
43. Laitila A, Sweins H, Vilpola A, Kotaviita E, Olkku J, Home S, Haikara A (2006) *Lactobacillus plantarum* and *Pediococcus pentosaceus* starter cultures as a tool for microflora management during malting process and for enhancement of malt processability. J Agric Food Chem (in Press)
44. Loureiro (2000) Spoilage yeasts in foods and beverages. Food Res Int 33:247–256
45. Loureiro V, Querol A (1999) The prevalence and control of spoilage yeasts in foods and beverages. Trends Food Sci Technol 10:356–365
46. Lowe D, Arendt E (2004) The use and effects of lactic acid bacteria in malting and brewing with their relationships to antifungal activity, mycotoxins and gushing: a review. J Inst Brew 110:163–180
47. Matos T, de Hoog G, de Boer A, de Crom I, Haase G (2002) High prevalence of the neurotrope *Exophiala dermatidis* and related oligotroph black yeasts in sauna facilities. Mycoses 45:373–377
48. Nichols A, Leaver C (1961) Methods of examining damp grain at harvest and after sealed and open storage: changes in the microflora of damp grain during sealed storage. J Appl Bacteriol 29:566–581
49. Noots I, Delcour J, Michiels C (1999) From field barley to malt: detection and specification of microbial activity for quality aspects. Crit Rev Microbiol 25:121–153
50. Noots I, Derycke V, Jensen H, Michiels C, Delcour J, Coppens T (2003) Studies on barley starchy endosperm cell wall degradation by *Rhizopus* VII. J Cereal Sci 37:81–90
51. O'Sullivan T, Walsh Y, O'Mahony A, Fitzgerald G, van Sinderen D (1999) A comparative study of malthouse and brewhouse microflora. J Inst Brew 105:55–61
52. Petters H, Flannigan B, Austin B (1988) Quantitative and qualitative studies of the microflora of barley malt production. J Appl Bacteriol 65:279–297
53. Pitt J, Hocking A (1997) Fungi and food spoilage. Blackie Academic & Professional, Cambridge, UK
54. Ruivo C, Lachance M, Rosa C, Bacci M Jr, Pagnocca F (2006) *Candida heliconiae* sp. nov., *Candida picinguabensis* sp. nov. and *Candida saopaulonensis* sp. nov., three ascomycetous yeasts from *Heliconia velloziana* (Heliconiaceae). Int J Syst Evol Microbiol (in press)
55. Saligkarias I, Gravanis F, Epton H (2002) Biological control of *Botrytis cinerea* on tomato plant by the use of epiphytic yeasts *Candida guilliermondii* strains 101 and US 7 and *Candida oleophila* strain I-182. *in vivo* studies. Biol Cont 25:143–150

56. Sarlin T, Laitila A, Pekkarinen A, Haikara A (2005) Effects of three *Fusarium* species on the quality of barley and malt. J Am Soc Brew Chem 63:43–49
57. Sarlin T, Nakari-Setalä T, Linder M, Penttilä M, Haikara A (2005) Fungal hydrophobins as predictors of the gushing activity of malt. J Inst Brew 111:105–111
58. Schauer F, Hanschke R (1999) Taxonomy and ecology of the genus *Candida*. Mycoses 42:12–21
59. Schena l, Finetti M, Gallitelli D (2002) Molecular detection of strain L47 of *Aureobasidium pullulans*, a biocontrol agent of postharvest diseases. Plant Dis 86:54–60
60. Schwarz P, Casper H, Beattie S (1995) Fate and development of naturally occurring *Fusarium* mycotoxins during malting and brewing. J Am Soc Brew Chem 53:121–127
61. Schwarz P, Jones B, Steffenson B (2002) Enzymes associated with *Fusarium* infection of barley. J Am Soc Brew Chem 60:130–134
62. Stars A, South J, Smith N (1993) Influence of maltings microflora on malt quality. In: Proceeding of the European Brewing Conversation, Oslo, Norway, pp 103–110
63. Steinkraus K (1998) Bio-enrichment: production of vitamins in fermented foods. In: Wood B (eds) Microbiology of fermented foods. Blackie Academic and Professional, London, UK, pp 603–621
64. Strauss M, Jolly N, Lambrechts M, van Rensburg P (2001) Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J Appl Microbiol 91:182–190
65. Suh S-O, Blackwell M (2004) Three new beetle-associated yeast species in the *Pichia guilliermondii*. FEMS Yeast Res 5:87–95
66. Thomas K, Usher J (2001) Interactions between lactic acid bacteria and malting barley. In: Andrews M, Andrews ME, Humphry D (eds) Plant microbial interactions: positive interactions in relation to crop production and utilisation. The Association of Applied Biologist, Warwick, UK, pp 239–250
67. Thomas-Hall S, Watson K (2002) *Cryptococcus nyarrowii* sp. nov., a basidiomycetous yeast from Antarctica. Int J Syst Evol Microbiol 52:1033–1038
68. Tuomi T, Laakso S, Rosenqvist H (1995) Plant hormones in fungi and bacteria from malting barley. J Inst Brew 101:351–357
69. Tuomi T, Rosenqvist H (1995) Annual variation in the microflora of some varieties of Finnish malting barley. Agric Sci Finl 4:407–418
70. Van Nierop S, Cameron-Clarke A, Axcell B (2004) Enzymatic generation of factors from malt responsible for premature yeast flocculation. J Am Soc Brew Chem 63:108–116
71. Voragen G, Schols H, Marijs J, Rombouts F, Angelino S (1987) Non-starch polysaccharides from barley: structural features and breakdown during malting. J Inst Brew 93:202–208
72. Yin X, MacGrecor A, Clear A (1989) Field fungi and β-glucan solubilase in barley kernels. J Inst Brew 95:195–198
73. Yurlova N, de Hoog G, Gerrits van den Ende A (1999) Taxonomy of *Aureobasidium* and allied genera. Stud Mycol 43:62–69
74. Zhao J-H, Bai F-Y, Guo L-D, Jia J-H (2002) *Rhodotorula pinicola* sp. nov., a basidiomycetous yeast species isolated from xylem of pine twigs. FEMS Yeast Res 2:159–163