



Brewing potential of the wild yeast species *Saccharomyces paradoxus*

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Abstract

Saccharomyces paradoxus is commonly isolated from environmental samples in Northern Europe and North America, but is rarely found associated with fermentation. However, as novelty has become a selling point in beer markets, interest toward non-conventional and local yeasts is increasing. Here, we report the first comprehensive investigation of the brewing potential of the species. Eight wild strains of *S. paradoxus* were isolated from oak trees growing naturally in Finland, screened in a series of fermentation trials and the most promising strain was selected for lager beer brewing at pilot scale (40 l). Yeasts were evaluated according to their ability to utilize wort sugars, their production of flavour-active aroma volatiles, diacetyl and organic acids, and sensorial quality of beers produced. All strains could assimilate maltose but this occurred after a considerable lag phase. Once adapted, most wild strains reached attenuation rates close to 70%. Adaptation to maltose could be maintained by re-pitching and with appropriate handling of the adapted yeast. Fermentation at 15 °C with the best performing strain was completed in 17 days. Maltose was consumed as efficiently as with a reference lager yeast, but no maltotriose use was observed. Bottled beers were evaluated by a trained sensory panel, and were generally rated as good as, or better than, reference beers. *S. paradoxus* beers were considered full-bodied and had a relatively clean flavour profile despite the presence of the clove-like 4-vinyl guaiacol. In conclusion, *S. paradoxus* exhibits a number of traits relevant to brewing, and with appropriate handling could be applied industrially.

Keywords *Saccharomyces paradoxus* · Lager beer · Fermentation · Aroma · Phenolic off flavour

Introduction

Saccharomyces paradoxus, despite being the closest relative of *S. cerevisiae*, is rarely encountered in beverage fermentations [1] and is considered to be a thoroughly wild yeast, without any of the traits typically associated with

domesticated yeasts [2, 3]. Its absence from fermentation systems, even as a contaminant, is interesting considering that it is one of the most abundant *Saccharomyces* species in Europe and in North America [4–6] and is found occasionally on vineyard grapes [7]. Its abundance in the wild, and paucity in fermentation environments, suggests that it is at a competitive disadvantage with respect to domesticated *Saccharomyces* species. The potential of *S. paradoxus* as a pure starter culture for wine fermentations has, however, been studied [8–11] and the results were promising, with good oenological properties including intense fruity and floral aromas. The brewing properties of *S. paradoxus* are unknown, though its ability to utilize maltose and abundance in the environment makes it an interesting candidate for brewing. The current trend in the brewing industry is for greater product diversity and novelty [12, 13], and brewers are interested in exploring the potential of alternative yeast strains for speciality beers and bioflavouring [14]. The successful application recently of the wild yeast *S. eubayanus* for industrial-scale brewing [15] suggests that there is

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a market demand for novel beers produced with alternative yeast.

Besides the already mentioned *S. cerevisiae*, *S. eubayanus* and *S. paradoxus*, the genus currently consists of five other species: *S. arboricola*, *S. jurei*, *S. kudriavzevii*, *S. mikatae*, and *S. uvarum* [16–21]. In addition to these, it is still under debate whether *S. cariocanus* can be considered a separate species or if it belongs to a South American population of *S. paradoxus* [22–24]. The genus has a worldwide distribution, but species-specific differences exist: *S. arboricola* and *S. mikatae* have not been isolated outside of Asia, and *S. eubayanus* has not yet been found in Europe. One complication related to isolation is that the ecological niches of *Saccharomyces* remain poorly understood. It has been proposed that oak is a natural niche [5], but there is often a sampling bias toward oak trees, and the *Saccharomyces* yeasts have been isolated elsewhere as well; Goddard and Greig [25] have proposed that the species *S. cerevisiae*, for example, could be nomadic by nature, i.e. found in low abundance in many different niches. Some of the distributional differences can be explained by the divergent temperature preferences of the species. *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* are both found in Europe, but apparently, the small deviation in optimal growth temperature enables the latter to inhabit cooler parts of Europe, making it the only natural wild *Saccharomyces* species found in northern Europe to date [26].

Several *Saccharomyces* species have been shown to possess some degree of fermentative ability [8, 27–30], but only two species are considered to include domesticated [31] strains: *Saccharomyces cerevisiae*—commonly found in different kinds of fermentations worldwide and with a domestication history of at least several hundred years [32]—and *Saccharomyces uvarum*—with a narrower distribution and most commonly found in wine and cider fermentations [18, 33]. Thus, the remaining species can be considered as wild, though *S. eubayanus*, *S. kudriavzevii* and *S. paradoxus* have been found in domesticated hybrids with *S. cerevisiae* [3, 20, 34]. The biggest flaw in wild species with respect to brewing seems to be their tendency to produce vinyl phenols,

like 4-vinyl guaiacol [30, 35, 36], which are considered off-flavours in many beer styles, limiting their wider usability in brewing. However, in some special beer styles, like wheat beers and saisons, these phenolic flavours are considered an essential part of the flavour profile.

In this study, *S. paradoxus* strains were isolated from natural stands of oak (*Quercus robur*) in south and south-west Finland, the most northerly points in Europe where oak occurs naturally. Brewing characteristics of eight strains were evaluated and compared with domestic brewing strains as well as the type strains of *S. paradoxus* and *S. cariocanus*. The most promising strain was selected for pilot-scale evaluation of fermentation performance and beer quality.

Materials and methods

Sampling

Environmental samples were obtained during May–July 2016 in oak woods in Southern Finland, between Helsinki and Turku (Table 1). The samples were collected aseptically using sterile swabs dipped in 85% glycerol, rubbed gently against the bark before transfer to sterile 15-ml tubes. Enrichment medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, sucrose 1%, ethanol 7.6%, 1 M HCl 0.1%, chloramphenicol 0.0001% [37]) was added and samples were incubated statically at 21 °C. When growth was apparent, i.e. the medium became turbid, 80 µl of mixed sample was plated on Sniegowski agar selection media [37] and incubated at the same temperature. Colonies with typical *Saccharomyces* morphology—cream/white in colour; smooth, non-powdery surface—were transferred to YPD plates for further identification.

ITS sequencing

Identification of the isolates was based on internal transcribed spacer (ITS) sequences. PCR analysis of the ITS1-5.8S-ITS2 region of the ribosomal DNA (rDNA) region was

Table 1 List of *S. paradoxus* wild isolates used in the study

Isolate	VTT culture collection code	Isolation date	Location	Coordinates
Sp3	VTT C-16968	17.5.2016	Oittaa, Espoo	60° 15' 49.6" N 24° 41' 22.6" E
Sp5	VTT C-16970	17.5.2016	Oittaa, Espoo	60° 15' 49.6" N 24° 41' 22.6" E
Sp6	VTT C-16971	24.5.2016	Lenholmen, Parainen	60° 14' 45.5" N 22° 13' 07.1" E
Sp8	VTT C-16973	24.5.2016	Tamminiemi, Naantali	60° 26' 41.6" N 22° 03' 20.8" E
Sp12	VTT C-16976	21.6.2016	Västerby, Tammisaari	59° 59' 05.5" N 23° 24' 54.4" E
Sp17	VTT C-16978	9.6.2016	Oittaa, Espoo	60° 14' 29.0" N 24° 40' 25.6" E
Sp20	VTT C-16981	21.6.2016	Koppnäsudden, Hanko	59° 50' 37.9" N 22° 58' 18.0" E
Sp23	VTT C-16983	4.7.2016	Otaniemi, Espoo	60° 11' 22.4" N 24° 49' 32.0" E

performed using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). DNA was extracted with Yeast DNA Extraction kit (Thermo Fisher Scientific). ITS sequence was amplified by PCR, and amplicons were purified with MinElute PCR Purification Kit (Qiagen, Netherlands) and sequenced at SeqLab (Germany). BLAST searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) in the NCBI nucleotide (nt) database were performed with the obtained sequences to identify the isolates.

Wort preparation

The wort for the fermentations was produced in the VTT pilot brewery. Milled pilsner malt was added to local Espoo City water in infusion mashing (mashing-in at 48 °C; temperature steps: 48 °C 30 min–63 °C 30 min–72 °C 30 min–78 °C 10 min); mash was filtered with Meura (Belgium) and boiled 60 min with Magnum hop pellets (alpha 15%). The strength of the all-malt wort was 15 °P and the target IBU level was 50. The wort was collected hot (over 90 °C) in stainless steel kegs and stored at 0 °C before the use. When required, the wort was diluted to lower strengths using sterile, de-aerated Espoo City water. Prior to 2 l and 40 l fermentations, the wort was aerated to 10 ppm of oxygen.

Strains

Eight *S. paradoxus* isolates were selected for small-scale fermentations (see Table 1). In addition to wild isolates, the following species were used as reference strains: *S. paradoxus* type strain (VTT C-09850), *S. cariocanus* type strain (VTT C-15951), lager yeast A15 (VTT A-63015) and ale yeast A62 (VTT A-81062) obtained from the VTT Culture Collection.

Fermentations

For the small-scale fermentations, yeasts were first propagated aerobically in liquid YPD (1% yeast extract, 2% peptone, 2% glucose) medium at 20 °C with shaking (80 rpm). After 48 h, yeast cells were collected by centrifugation. Cell number and viability were determined through propidium iodide staining and fluorescence microscopy with the aid of the NucleoCounter YC-100 (ChemoMetec, Denmark). Cell suspensions of 1×10^9 viable cells ml^{-1} were prepared. Pitching of small-scale fermentations was carried out at a rate of 1×10^7 viable cells ml^{-1} and the fermentations were carried out in 100 ml of 15 °P wort at 20 °C in 250-ml Erlenmeyer flasks fitted with airlocks (filled with sterile glycerol 85%), and shaken (80 rpm). Fermentation was monitored as CO_2 loss by measuring the mass of the vessels at intervals

of 1 to 3 days for a period of up to 617 h depending on the strain. All fermentations were conducted in duplicate. At the end, the yeasts were retained for subsequent fermentations.

An inoculation loop was used to transfer a small amount of yeast from the end of the small-scale fermentations into fresh YPM (1% yeast extract, 2% peptone, 2% maltose) and propagated under aerobic conditions on a shaker (80 rpm) at 20 °C. After propagation (48 h), cell number and viability were determined. Cell suspensions of 1×10^9 viable cells ml^{-1} were prepared. Pitching of fermentations was carried out at a rate of 1×10^7 viable cells ml^{-1} . Fermentations were conducted at 2 l scale in sterile stainless steel cylindroconical vessels under two different conditions: 15 °P wort at 15 °C and 12 °P wort at 20 °C. Samples were taken aseptically throughout the fermentation period. In the end, the yeasts were cropped once again for further use.

For 40-l-scale fermentations, yeast propagation was performed similarly as for 2-l-scale fermentations. After propagation, yeast was collected, cell number determined with NucleoCounter YC-100 and 8 l, generation 0 (G0), fermentations were pitched with propagated yeast at a pitching rate 1×10^7 viable cells ml^{-1} . After 7 days of G0-fermentation, cells were collected, viability was determined, and 40-l-scale fermentations were pitched at a rate of 1×10^7 viable cells ml^{-1} . Fermentations were carried out in sterile, stainless steel cylindroconical vessels in 15 °P at 15 °C with regular measurements as outlined above.

Green beers were matured at +10 °C for 10 days, before 5-day stabilization at 0 °C, after which the beers were depth filtered (Seitz EK, Pall Corporation, USA) and adjusted to 4.5% ABV using sterile, de-aerated Espoo City water. Finally, the beers were carbonated to 5 g l^{-1} of CO_2 and bottled.

Wort and beer analyses

The samples for monitoring the fermentations were centrifuged and supernatants used for wort/beer analyses after manual degassing. The specific gravity, alcohol level (% v/v) and pH of samples were determined from the centrifuged and degassed wort and fermentation samples using an Anton Paar Density Meter DMA 5000 M with Alcolyzer Beer ME and pH ME modules (Anton Paar GmbH, Austria).

The yeast fresh mass content of the samples (i.e. yeast in suspension) was determined by washing the yeast pellets gained from centrifugation twice with 25 ml deionized H_2O in a pre-weighed centrifuge tube and calculating the mass of the pellet after removal of supernatant. Where necessary, the centrifuged and re-suspended yeast were analysed for cell viability using the Chemometec Nucleocounter as described above.

For further analyses, the samples collected at the end of the fermentation were centrifuged and the supernatants were

filtered (0.45 μm) prior to storing in a freezer ($-23\text{ }^{\circ}\text{C}$). Sugar content of wort was analysed by HPLC. A Waters 2695 Separation Module and Waters System Interphase Module liquid chromatograph coupled with a Waters 2414 differential refractometer (Waters Co., Milford, MA, USA) were used. An Aminex HPX-87H Organic Acid Analysis Column (300 \times 7.8 mm; Bio-Rad, USA) was equilibrated with 5 mM H_2SO_4 (Titrisol, Merck, Germany) in water at $55\text{ }^{\circ}\text{C}$, and samples were eluted with 5 mM H_2SO_4 in water at a 0.3 ml min^{-1} flow rate.

Yeast-derived flavour compounds were determined by headspace gas chromatography with flame ionization detector (HS-GC-FID) analysis. 4 ml samples was filtered (0.45 μm), incubated at $60\text{ }^{\circ}\text{C}$ for 30 min and then 1 ml of gas phase was injected (split mode; $225\text{ }^{\circ}\text{C}$; split flow of 30 ml min^{-1}) into a gas chromatograph equipped with an FID detector and headspace autosampler (Agilent 7890 Series; Palo Alto, CA, USA). Analytes were separated on an HP-5 capillary column (50 m \times 320 μm \times 1.05 μm column, Agilent, USA). The carrier gas was helium (constant flow of 1.4 ml min^{-1}). The temperature program was $50\text{ }^{\circ}\text{C}$ for 3 min, $10\text{ }^{\circ}\text{C min}^{-1}$ to $100\text{ }^{\circ}\text{C}$, $5\text{ }^{\circ}\text{C min}^{-1}$ to $140\text{ }^{\circ}\text{C}$, $15\text{ }^{\circ}\text{C min}^{-1}$ to $260\text{ }^{\circ}\text{C}$ and then isothermal for 1 min. Compounds were identified by comparison with authentic standards and were quantified using standard curves. 1-Butanol was used as internal standard.

Total vicinal diketones (VDKs, free and acetohydroxy acid form) were measured for the centrifuged fermentation samples according to Analytica-EBC method 9.10 (European Brewery Convention 2008). Samples were heated to $60\text{ }^{\circ}\text{C}$ (heating to $60\text{ }^{\circ}\text{C}$ results in the conversion of acetohydroxy acids to VDKs), where they were kept for 120 min in a water bath before analysis by HS-GC/ECD. The samples were then analysed by headspace gas chromatography (Headspace Autosampler: Agilent 7697A, USA and Agilent 7890B gas chromatography, USA; HP-5 50 m \times 320 μm \times 1.05 μm column, Agilent, USA) with 2,3-hexanedione as an internal standard.

Organic acids were determined in duplicate using a P/ACE MDQ capillary electrophoresis instrument equipped with a photodiode array UV–Vis detector working by indirect detection at a wavelength of 230 nm (Beckman-Coulter Inc., Fullerton, USA). For organic acid detection and quantification, standards were prepared as described and analysed prior to analyses of the actual samples. Electrolyte solutions were commercial from Analis [Ceofix Anions 5 (Ordior/Analis, PART NUMBER: 10-0004650)]. Bare fused silica capillary with inner diameter 75 μm , the detector length to the UV detector was 50 cm and the total length was 60 cm. The separation voltage of -30 kV and polarity of positive to negative were applied. Samples were injected using 0.5 psi pressure for 8 s. Separation of organic acids was carried out after diluting the sample by capillary electrophoresis. The standard stock

solutions for each acid were 1000 mg l^{-1} . For the calibration, each stock solution was mixed and diluted to obtain proper concentration range for quantification (1, 5, 10, 25, 50 and 100 mg l^{-1}). On the basis of the corresponding standard compounds, formic, acetic, lactic, propionic and butyric acid were quantified (mg l^{-1} of sample).

Statistical analysis was performed on the fermentation data with one-way ANOVA and Tukey's test using the "agricolae" package in R (<https://www.r-project.org/>).

Sensory analysis

For small-scale fermentations, preliminary sensory analysis was performed simply by smelling the green beers after the yeast was separated in centrifuge (9000 rpm, $1\text{ }^{\circ}\text{C}$, 10 min) and the supernatant (i.e. the green beer) transferred to a capped 50 ml test tube. Beers were assessed at room temperature by a sensory panel of three persons.

For the bottled pilot-scale beers, the beer samples were tasted and judged by a commercial brewery in-house sensory panel of ten experienced beer tasters. Beer flavour profiling created by Meilgaard [38] was used for tasting. Panellists were asked to evaluate attribute intensity on a scale from 0, "nothing," to 5, "extreme." Samples, at a temperature of $10\text{ }^{\circ}\text{C}$, were provided in glasses, and tested blind, with a four-digit code used for identification.

Yeast pre-conditioning

To assess the impact of pre-conditioning on fermentation performance, single cells from yeast slurry cropped from 2-l-scale fermentations were isolated using a Singer MSM 400 Micro-manipulator. This was done on both regular YPD agars and YNB–maltose agars (0.67% yeast nitrogen base without amino acids, 2% maltose) and plates were incubated under anaerobic conditions until colonies appeared. DNA was isolated and ITS-PCR identification was performed as described above to confirm that the colonies belonged to the original culture. One colony from a YPD plate was propagated in liquid YPD (50 ml) and the colony from YNB–maltose plate was propagated in liquid YPM (50 ml) for 48 h at $21\text{ }^{\circ}\text{C}$. The propagated yeasts were then used to inoculate autoclaved in-house all-malt wort at a strength of $12\text{ }^{\circ}\text{P}$ (diluted from $15\text{ }^{\circ}\text{P}$ wort with Espoo City water prior to autoclaving) at a pitching rate of 2 g l^{-1} . The static fermentations (100 ml; in a Schott bottle; capped with airlock filled with 85% glycerol) with duplicates were monitored by regular weighing.

Results

Isolations

The total number of samples collected was 283, which were obtained from 16 sampling sites. After morphology-based pre-selection, the ITS regions of 48 isolates were sequenced and sorted with MultAlin (<https://multalin.toulouse.inra.fr/multalin/>). The sequencing reads of 30 isolates were identical to each other (a common span of 733 bp nucleotides in the middle of the sequences, though they varied in length: from 753 to 789 bp) having a 100% query coverage and 100% identity with the best hit, *Saccharomyces paradoxus* NRRL Y-17217, in a BLAST search of the NCBI Nucleotide database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Thus, the samples yielded 30 *S. paradoxus* strains, which were distributed evenly between locations and only two locations had no positive results. No other *Saccharomyces* species were detected in the samples.

Small-scale fermentations

Fermentations were conducted with 8 *S. paradoxus* strains isolated from different geographical areas (Table 1) at 100-ml scale in 15 °P wort at 20 °C and the strains of *S. pastorianus* A15, *S. cerevisiae* A62, *S. paradoxus* type strain C-09850 and *S. cariocanus* type strain C-15951 were included as controls. Mass loss during small-scale fermentations was monitored (Fig. 1). When there was no change in mass during consecutive sampling days, fermentations were regarded as finished and beer was collected. This took place 162 h after pitching for A15, A62 and the

S. paradoxus type strain. *S. cariocanus* and wild isolates fermented more slowly compared to the other strains. Most isolates reached the mass loss values obtained with the *S. paradoxus* type strain, albeit after a longer period of time. *S. cariocanus* and wild *S. paradoxus* isolates had long lag phases, often extending over 300 h before a clear drop in mass was observed. This delay considerably extended the fermentation time for these strains. The Sp3 isolate and *S. cariocanus* strain had shorter lag times compared to other wild isolates. With these fermentations, no considerable mass loss was observed after the 420 h time point and the fermentations were stopped at 498 h. All remaining fermentations were stopped at 617 h. Apart from the slowly fermenting wild strains Sp20 and Sp23, the ABV values obtained for wild isolates (from 5.7 to 5.9%) were comparable to the type strains of *S. paradoxus* (ABV 5.9%) and *S. cariocanus* (ABV 5.9%), but clearly lower than the values from the conventional brewing yeasts A15 and A62 (ABV 7.0% and 7.4%, respectively). There were relatively large differences in viability between the wild isolates. Sp3 and Sp17 were the weakest having only 63% and 69% viability, respectively, after fermentations (Table 2).

The concentrations of aroma-active higher alcohols and esters were considerably lower in the beers produced with the *S. paradoxus* strains compared to the industrial brewing strains (Fig. 2). The beers produced with the wild isolates of *S. paradoxus* were similar to each other in their aroma profiles (with the exception of beer produced by Sp23 isolate). Preliminary sensory analysis by three persons found Sp3, Sp6 and Sp17 isolates to be the most pleasant sensorially. These three isolates were chosen for further evaluation based on their sensory attributes and their fermentation performance.

Fig. 1 Relative mass (%) in 100 ml fermentations. Values are means of two independent replicates. Due to high deviation between the replicates, the error bars have been left out

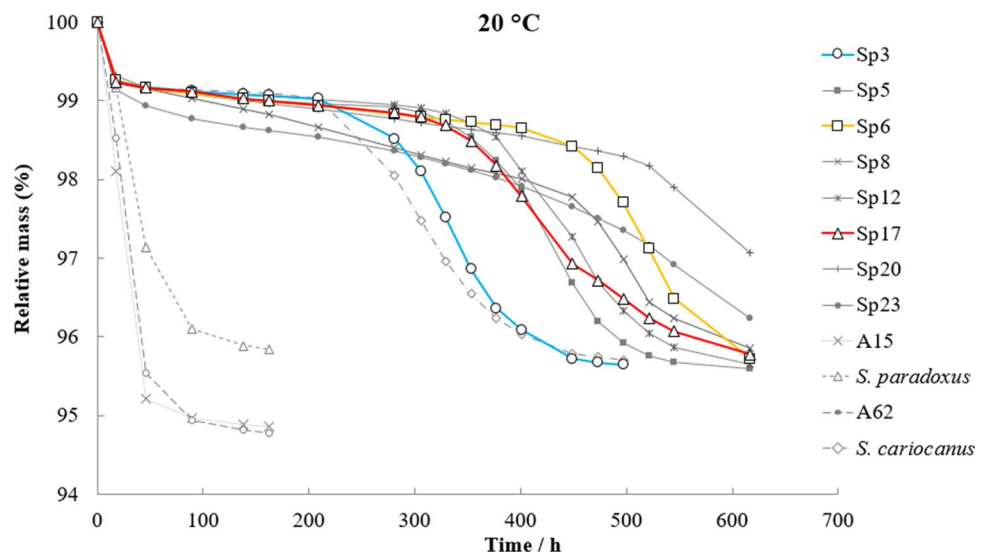


Table 2 Fermentation characteristics of *S. paradoxus* wild isolates and reference strains used to ferment all-malt 15 °P wort

	Sp3	Sp5	Sp6	Sp8	Sp12	Sp17	Sp20	Sp23	Lager A15	<i>S. paradoxus</i> C-09850	Ale A62	<i>S. cariocanus</i> C-15951
Alcohol by volume (%)	5.9±0.0 ^{ab}	5.8±0.0 ^{ab}	5.8±0.4 ^{ab}	5.7±0.4 ^{ab}	5.8±0.0 ^{ab}	5.7±0.8 ^{ab}	4.0±0.3 ^{ab}	3.1±3.1 ^b	7.0±0.1 ^a	5.9±0.0 ^{ab}	7.4±0.0 ^a	5.9±0.0 ^{ab}
°Plato	4.1±0.3	4.0±0.0	4.1±0.8	4.5±0.8	4.0±0.3	4.4±1.5	7.4±0.7	6.9±2.3	1.7±0.0	3.9±0.1	1.1±0.0	4.0±0.1
pH	4.4±0.0 ^{ab}	4.5±0.0 ^{ab}	4.4±0.0 ^{ab}	4.4±0.0 ^{ab}	4.3±0.1 ^b	4.3±0.0 ^b	4.4±0.0 ^{ab}	4.3±0.1 ^b	4.6±0.0 ^a	4.4±0.0 ^{ab}	4.2±0.1 ^b	4.3±0.0 ^b
Yeast fresh mass (g l ⁻¹)	31.3±1.6	29.6±1.8	30.9±0.5	25.4±0.5	28.9±0.7	29.1±1.1	28.7±0.1	40.2±17.3	40.4±2.5	38.5±1.2	37.7±4.4	30.7±1.8
Viability (%)	63±1 ^{ab}	75±11 ^{ab}	87±3 ^{ab}	84±10 ^{ab}	73±2 ^{ab}	69±19 ^{ab}	88±2 ^{ab}	76±21 ^{ab}	81±7 ^{ab}	81±3 ^{ab}	51±10 ^b	95±1 ^a
Flocculation ability ^A	—	—	—	—	—	—	—	—	—	—	+++	++
Fermentation time (h)	498	617	617	617	617	617	617	617	162	162	162	498

^ASymbols: — no flocculation, + faint flocculation, + strong flocculation, +++ very strong flocculation

Fermentations were conducted at 100-ml scale at 20 °C. Values are mean of two independent replicates. The range is included. Values in the same row with different superscript letters differ significantly ($p < 0.05$) as determined by one-way ANOVA and Tukey's test

2-l-Scale fermentations

2-l-Scale wort fermentations were inoculated with yeast collected from the small-scale fermentations. In contrast to the small-scale fermentations, where the yeast had been propagated on glucose media, no lag phase was observed and all fermentations started without any delays. The fermentations at 2-l scale were performed at two different temperatures to test the temperature tolerance of the wild isolates. Low-temperature fermentations were conducted at 15 °C in 15 °P wort and high-temperature fermentations were conducted at 20 °C in 12 °P wort. At 20 °C, A15 fermentations were rapid, with apparent extract of 1.6 °P (5.4% ABV) achieved after only 72 h (Fig. 3c). The ale strain A62 reached a lower apparent extract (1.0 °P) than A15, but only after 250 h of fermentation. The *S. paradoxus* strains, type strains and wild isolates, fermented more slowly and reached apparent extract values of 3.0 °P to 3.4 °P (ABV values from 3.0 to 3.4%) only by 359 h. Sp3 and Sp17 were the best performing *S. paradoxus* strains. Viability of yeast after fermentations conducted at 20 °C was poor for Sp3, being only 36%. Viabilities for other yeast were between 67 and 88% (Fig. 3d).

Fermentations conducted at 15 °C took more time to complete compared to those carried out at 20 °C. A15 fermentations reached an apparent extract value of 2.5 °P (6.8% ABV) at 237 h (Fig. 3a). A62 fermented slower than A15, and reached an apparent extract value of 1.6 °P (7.4% ABV) after 765 h. The lower fermentation temperature of 15 °C appeared to be challenging for two of the wild isolates, Sp6 and Sp17, and also for the type strain of *S. paradoxus*. An increasing deviation between the fermentation duplicates with Sp6 and Sp17 was observed after 25% of wort sugars were consumed. With the *S. paradoxus* C-09850 type strain, there was a long, additional lag phase and only after 450 h did fermentation proceed more efficiently, reaching a final ABV value of 5.9%. Sp3 performed clearly better than the two other isolates, reaching an ABV of 5.7% compared to the 4.1% and 3.8% values reached by Sp6 and Sp17, respectively. Viabilities of the yeasts were generally higher after fermentations conducted at 15 °C compared to those performed at 20 °C. For example, Sp3 had a poor viability (36%) at 20 °C compared to 93% at 15 °C. Also, other yeasts had higher viabilities (77–98%) at 15 °C compared to those at 20 °C (67–88%). The exception was the wild isolate Sp17, which showed similar results (83% and 88%) at both temperatures (Fig. 4).

Sp3 was clearly the best performing isolate at both temperatures, having a final ABV of 5.7% at 15 °C and 4.6% at 20 °C. There was no clear peak in the fresh or dry mass values at either temperature suggesting that no distinct flocculation occurred with wild *S. paradoxus* strains (data not shown).

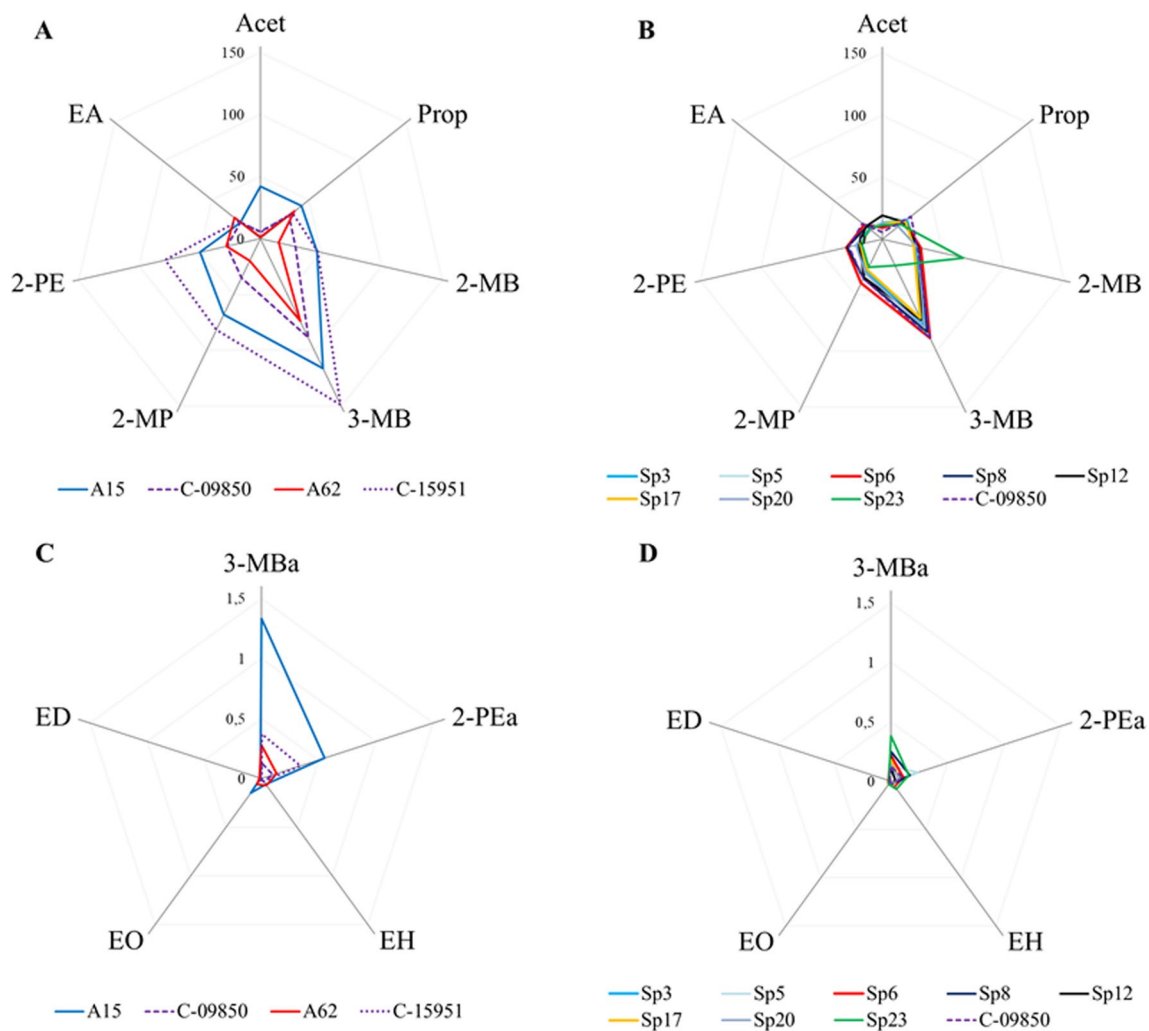


Fig. 2 Radar charts of acetaldehyde, ethyl acetate and higher alcohols (charts **a**, **b**) and acetate and ethyl esters (**c**, **d**) in beers at the end of 100 ml fermentations. Values (mg l^{-1}) are mean of two independent replicas. **a**, **c** Values for three controls and **b**, **d** for wild isolates. To facilitate comparison, the values of *S. paradoxus* type strain (C-09850) are included in both charts (dotted line). *Acet* acetalde-

hyde, *Prop* propanol, *2-MB* 2-methylbutanol, *3-MB* 3-methylbutanol, *2-MP* 2-methylpropanol, *2-PE* 2-phenylethanol, *EA* ethyl acetate, *3-MBa* 3-methylbutyl acetate, *2-PEa* 2-phenylethyl acetate, *EH* ethyl hexanoate (ethyl caproate), *EO* ethyl octanoate (ethyl caprylate), *ED* ethyl decanoate; for numerical values, please see supplementary material S1

The lower fermentation temperature had a different effect on the aroma compound productivity with different yeasts: Sp3 produced more esters and higher alcohols at 15 °C compared to 20 °C, whereas the levels were decreased with the rest of the *S. paradoxus* strains; with the reference brewing strains, the levels of higher alcohols were decreased, but esters increased. The biggest relative increases of single compounds with Sp3 strain were in ethyl acetate (fruity, solvent; from 18.8 to 30.1 mg l^{-1}) and ethyl hexanoate (pineapple; from 0.21 to 0.36 mg l^{-1}) and these values were comparable to the ale yeast A62 (34.1 mg l^{-1} and 0.4 mg l^{-1} , respectively). *S. paradoxus* type strain produced high levels of higher alcohols at 20 °C, having peak values of all the strains with 2- and 3-methyl butanol and 2-methyl

propanol, but the ester levels at 20 °C were below those of the reference strains. Lowering temperature reduced the levels of aroma compounds with the type strain and the only increase was seen with ethyl octanoate values (from 0.1 to 0.47 mg l^{-1}) (Table 3).

40 l fermentations

During the final phase of the study, pilot-scale fermentations were carried out with the best performing *S. paradoxus* isolate, Sp3, isolated from Oittaa, Espoo (Table 1). Fermentations were carried out at 15 °C and the lager yeast strain A15 was included for comparison. Lager-style fermentations conducted at 15 °C took around 2 weeks, *S.*

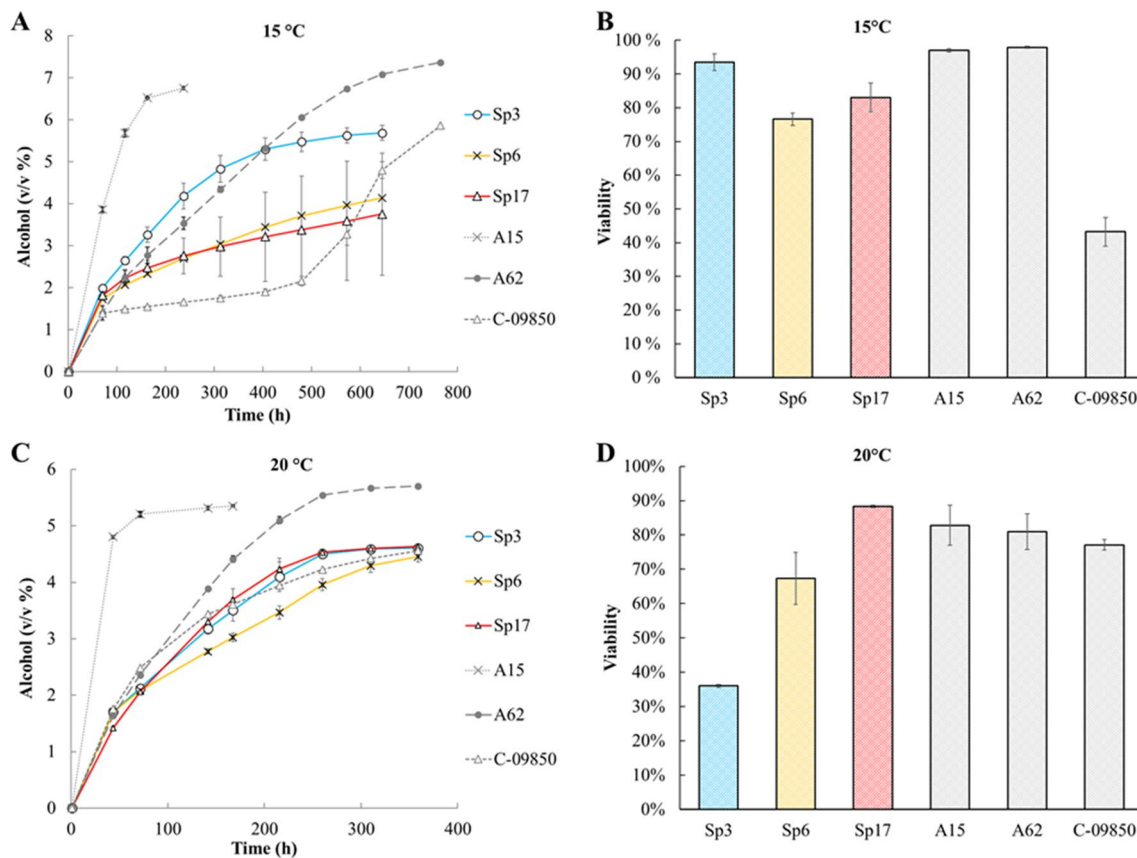


Fig. 3 **a** Fermentation profiles of 2-l-scale fermentations conducted in 15 °P wort at 15 °C for up to 765 h and **b** the viability rates at the end. **c** Fermentation profiles in 2-l-scale fermentations conducted in 12 °P wort at 20 °C fermented for up to 359 h and **d** viability rates at

the end. All values are means of two independent fermentations and error bars, when visible, represents the deviation between the parallels

pastorianus A15 fermentations reached apparent extract of 2.5 °P (7.0% ABV) after 310 h (13 days) and Sp3 fermentations reached an apparent extract of 4.2 °P (6.0% ABV) after 406 h (17 days). The sugar contents of the green (i.e. non-matured) beers are presented in Table 4, and clearly show that Sp3 yeast was not able to utilize maltotriose.

The *S. paradoxus* isolate Sp3 maintained its viability to some extent only in suspension (viability 68%), while the viability of sedimented yeast was only 9% (Fig. 5b), indicating that there was apparently no true flocculation with Sp3, but rather sedimentation of non-viable cells. This can also be seen in dry-mass values (Fig. 5d), in which the Sp3 value remains high till the end of the fermentation. In comparison, the A15 yeast had a viability of 93% in suspension and 91% in cone (sediment), and its dry-mass curve has a clear peak.

Aroma volatile levels in A15 green beer were greater than in Sp3 beers (Fig. 6a, b), especially with the acetate esters: 3-methylbutyl acetate (banana, pear) and 2-phenylethyl acetate (rose, honey). Green beer of Sp3 had three aroma values higher than A15: propanol (12.4 mg l⁻¹ and 11.5 mg l⁻¹, respectively), 2-methylpropanol (14.6 mg l⁻¹

and 11.7 mg l⁻¹, respectively; aroma notes: apple, cocoa) and ethyl hexanoate (0.35 mg l⁻¹ and 0.24 mg l⁻¹, respectively). After the maturation and stabilization steps, the beers were diluted to 4.5% ABV, involving a greater dilution of the stronger A15 beer (38% of water for A15 and 25% for Sp3). After conditioning, dilution and bottling, the concentrations of higher alcohols and esters were decreased to 32% and 48% in the A15 and Sp3 beers, respectively (Fig. 6c, d). Diacetyl concentrations in green and bottled beers were 25 mg l⁻¹ and 20 mg l⁻¹, respectively, for Sp3 beer and 34 mg l⁻¹ and 12 mg l⁻¹, respectively, for A15 beer (Table 5).

The wild yeast Sp3 had a lower pH value (Fig. 5c) and it produced more acetic, lactic and propionic acid than lager yeast A15 (Fig. 7). Acetic acid concentration of Sp3 beer (192 mg l⁻¹) was slightly above the flavour threshold (175 mg l⁻¹, [39]). Propionic and lactic acid concentrations (109 mg l⁻¹ and 295 mg l⁻¹, respectively) were below threshold values. The corresponding values in A15 beer were propionic acid, 99 mg l⁻¹; acetic acid, 73 mg l⁻¹; lactic acid, 256 mg l⁻¹.

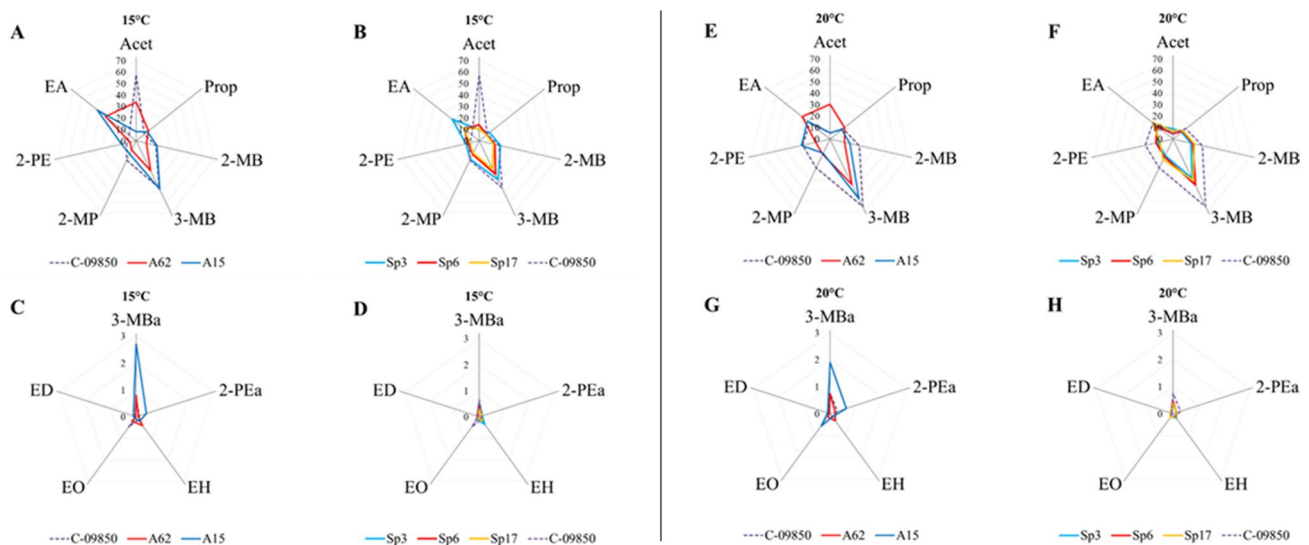


Fig. 4 Radar charts for aroma characteristics at the end of 2-l fermentations at 15 °C (charts **a–d**) and at 20 °C (charts **e–g**). Acetaldehyde, ethyl acetate and higher alcohol concentration (charts **a, b, e, f**). Acetate and ethyl ester concentrations at the end of 2-l fermentations (charts **c, d, g, h**). All values (mg l^{-1}) are mean of two independent replicas. In **a, c, e** and **g** values for control strains and in **b, d, f** and **g** values for the wild isolates are shown. To facilitate comparison between the charts, *S. paradoxus* values (dotted line)

are shown in both, control and wild isolate charts. *Acet* acetaldehyde, *Prop* propanol, *2-MB* 2-methylbutanol, *3-MB* 3-methylbutanol, *2-MP* 2-methylpropanol, *2-PE* 2-phenylethanol, *EA* ethyl acetate, *3-MBa* 3-methylbutyl acetate, *2-Pea* 2-phenylethyl acetate, *EH* ethyl hexanoate (ethyl caproate), *EO* ethyl octanoate (ethyl caprylate), *ED* ethyl decanoate; for numerical values, please see the supplementary material

Table 3 Fermentation characteristics of *S. paradoxus* wild isolates and reference strains used to ferment all-malt wort at 2-l scale in two different conditions

	Sp3	Sp6	Sp17	<i>S. paradoxus</i> C-09850	Ale A62	Lager A15
At 15 °C						
Alcohol by volume (%)	5.7 ± 0.2 ^{a,b,c}	4.1 ± 1.5 ^{b,c}	3.8 ± 0.7 ^c	5.9 ± 0.0 ^{a,b,c}	7.4 ± 0.0 ^a	6.8 ± 0.0 ^{a,b}
°Plato	4.6 ± 0.3	7.4 ± 2.6	8.1 ± 1.3	4.2 ± 0.0	1.6 ± 0.0	2.5 ± 0.0
pH	4.2 ± 0.0 ^c	4.3 ± 0.0 ^{b,c}	4.2 ± 0.0 ^{b,c}	4.3 ± 0.0 ^{b,c}	4.3 ± 0.0 ^b	4.6 ± 0.0 ^a
Viability (%)	93 ± 3 ^{a,b}	77 ± 2 ^c	83 ± 4 ^{b,c}	83 ± 2 ^d	98 ± 0 ^a	97 ± 1 ^a
Fermentation time (h)	645	645	645	765	765	237
Diacetyl ($\mu\text{g l}^{-1}$) ^A	19 ± 2	29 ± 4	29 ± 8	97 ± 30	194 ± 11	83 ± 3
At 20 °C						
Alcohol by volume (%)	4.6 ± 0.0 ^c	4.5 ± 0.1 ^c	4.6 ± 0.0 ^c	4.6 ± 0.0 ^c	5.7 ± 0.0 ^a	5.4 ± 0.0 ^b
°Plato	3.2 ± 0.0	3.4 ± 0.2	3.0 ± 0.0	3.1 ± 0.2	1.0 ± 0.0	1.6 ± 0.0
pH	4.3 ± 0.0 ^{a,b}	4.3 ± 0.0 ^{a,b}	4.3 ± 0.0 ^c	4.3 ± 0.0 ^{b,c}	4.2 ± 0.0 ^d	4.4 ± 0.0 ^a
Viability (%)	36 ± 0 ^c	67 ± 8 ^b	88 ± 0 ^a	77 ± 2 ^{a,b}	81 ± 5 ^{a,b}	83 ± 6 ^{a,b}
Fermentation time (h)	359	359	359	359	359	168
Diacetyl ($\mu\text{g l}^{-1}$) ^A	12 ± 1	16 ± 1	13 ± 0	13 ± 2	26 ± 3	13 ± 1

The 12 °P wort fermentations were conducted at 20 °C and 15 °P wort fermentations at 15 °C. Values are means of two independent replicates. The range is included. Values in the same row with different superscript letters differ significantly ($p < 0.05$) as determined by one-way ANOVA and Tukey's test

^AThreshold level for diacetyl is 100–200 $\mu\text{g l}^{-1}$ [39]

In a sensory panel test (Table 6), the Sp3 beer was found to be more estery (1.72 on a 0–5 scale) and more fruity (1.61) than A15 beer (0.79 and 0.97, respectively). Sp3 beer was also found to be hoppier and to have greater mouthfeel than A15, but as the beers had different

dilutions, comparing these attributes directly is difficult. Phenolic taste of Sp3 beer was evident (2.54), but other off-flavours, like fatty acids, sulphidic, and cooked vegetable notes were absent, though detected in the A15 reference beer at low levels.

Table 4 Sugar content (g l^{-1}) of 15 °P wort and green beers produced with 40-l-scale fermentations

	Maltotriose	Maltose	Glucose	Fructose
Wort	19.6 ± 0.3	57.0 ± 0.9	15.7 ± 0.5	4.5 ± 1.2
A15 beer	6.5 ± 0.3	2.8 ± 0.9	0.0	0.0
Sp3 beer	19.7 ± 0.2	2.6 ± 0.9	0.0	0.0

Yeast pre-conditioning

To validate the obtained fermentation results and to study the uptake of maltose further, single cells from the Sp3 yeast slurry were isolated and grown on YPD and YNB–maltose plates under anaerobic conditions. The single colony from YPD plate was propagated in liquid YPD and the colony on YNB–maltose plate was propagated in YPM prior to fermentations. Relative weight-loss curve (Fig. 8) of the strain grown on maltose was similar to those seen in 2 l scale and in 40 l scale, i.e. no additional lag phase was apparent, though the fermentation took longer and was ended after 22 days. Contrary to this, the strain grown on glucose had lost its readiness to utilize α -glucoside sugars and had the

additional lag phase after relative weight loss of 0.5%—comparable to the earlier small-scale fermentation curves. Also, the deviation between the duplicates with the glucose-grown strain increases steadily during the lag phase.

Discussion

Wild yeasts have received considerable attention in brewing yeast research in recent years. The discovery of *S. eubayanus* [20] and its successful industrial-scale application [15], bacterial-free methods for producing sour beers [40], and promising results with non-alcoholic and low-alcoholic beers [41] are just some of the reasons behind the growing interest in wild yeasts. In this study, we focused on *Saccharomyces* obtained from oak trees in Finland, one of the most northern areas where oak forests occur naturally in Europe, and assessed the brewing potential of eight isolated *S. paradoxus* strains.

Among the 283 samples collected from oaks in different geographical locations in Southern Finland, *Saccharomyces paradoxus* was the only identified *Saccharomyces* species. Their share, 30 isolates, makes the incidence of the species

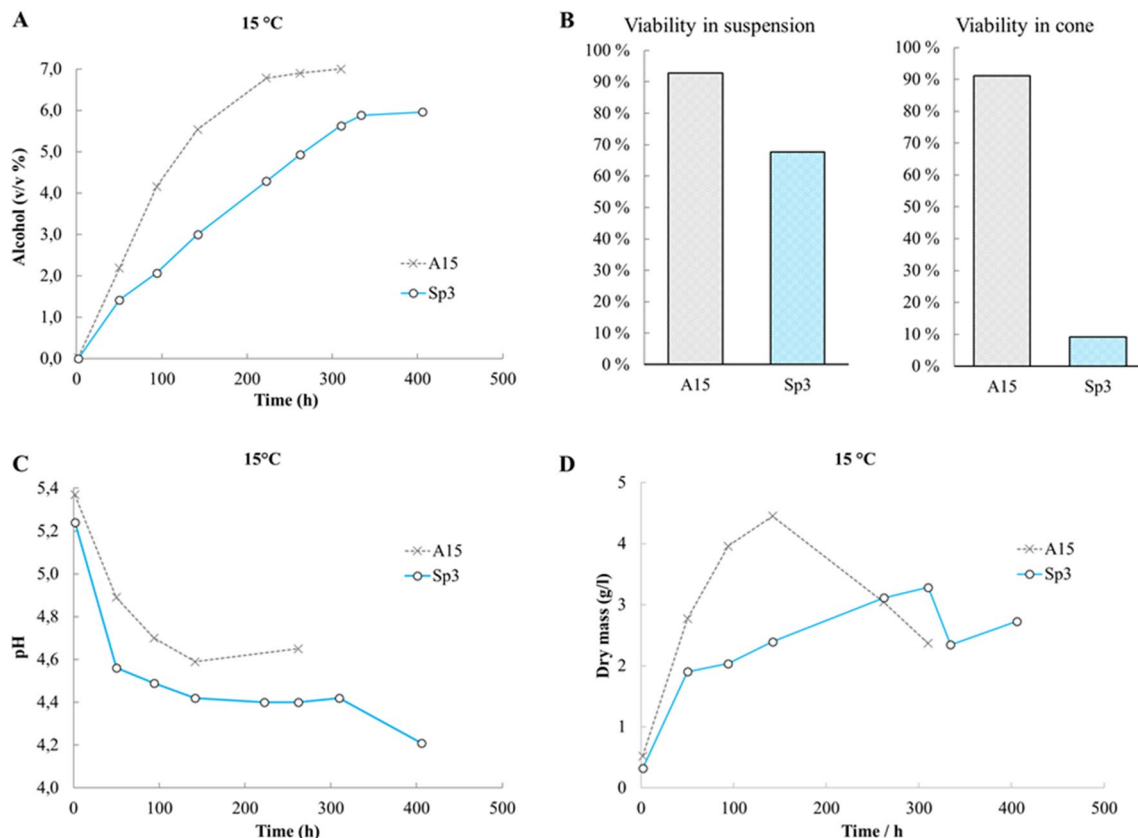


Fig. 5 Fermentation profiles of 40 l-scale fermentations conducted in 15 wort at 15 °C for 310 h (A15) and 406 h (Sp3). **a** ABV during the fermentation, **b** the final viabilities in suspension and in cone, **c** pH values and **d** the dry mass values

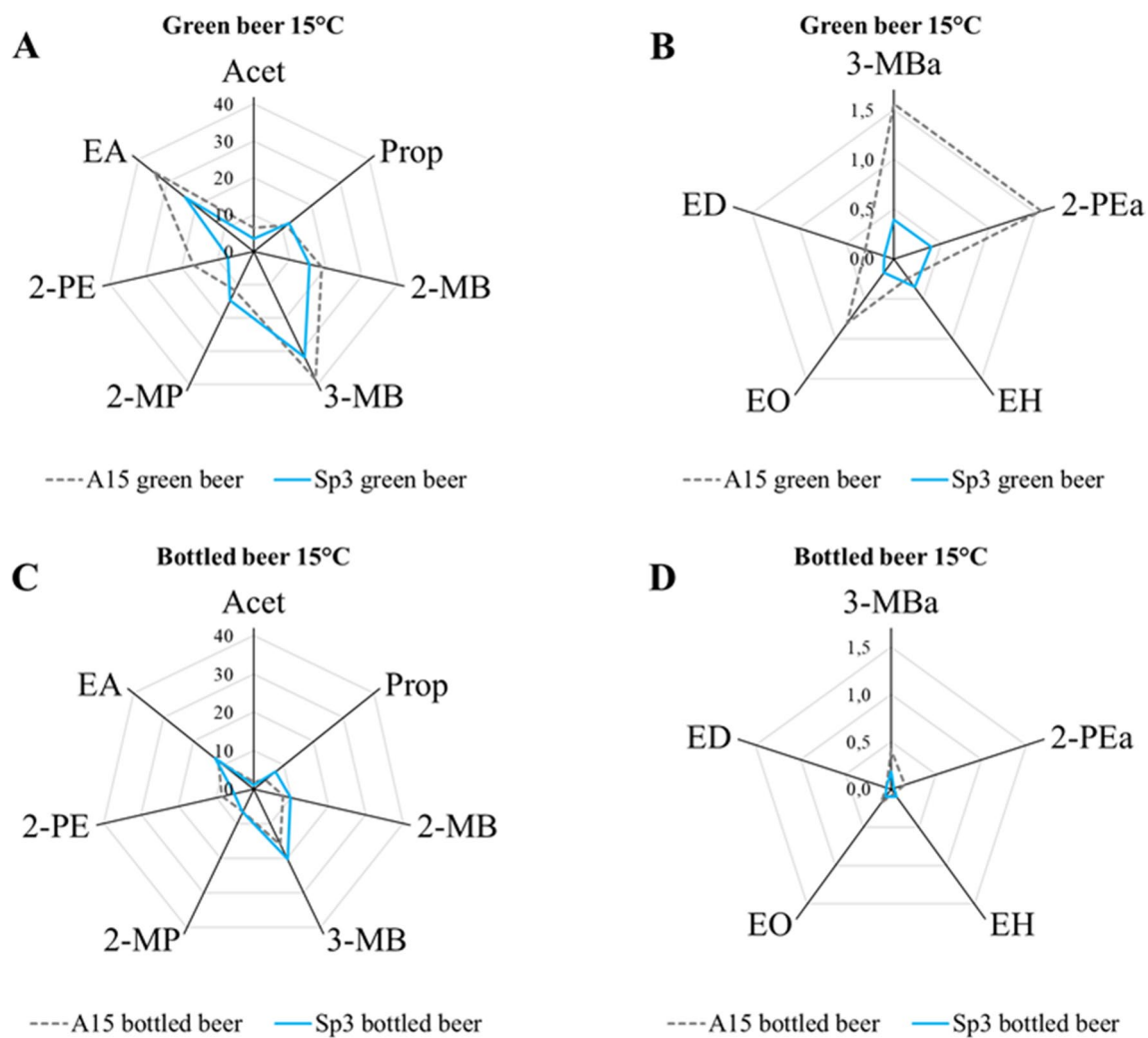


Fig. 6 Aroma characteristics of 40-l fermentations. Radar charts of acetaldehyde, ethyl acetate and higher alcohol concentrations (charts **a**, **c**), and acetate and ethyl ester concentrations (charts **b**, **d**) in green and bottled beers. Values are mg l^{-1} . *Acet* acetaldehyde, *Prop* propanol, *2-MB* 2-methylbutanol, *3-MB* 3-methylbutanol, *2-MP* 2-meth-

ylpropanol, *2-PE* 2-phenylethanol, *EA* ethyl acetate, *3-MBa* 3-methylbutyl acetate, *2-Pea* 2-phenylethyl acetate, *EH* ethyl hexanoate (ethyl caproate), *EO* ethyl octanoate (ethyl caprylate), *ED* ethyl decanoate; for numerical values, please see the supplementary material

Table 5 Fermentation characteristics of 40 l fermentations conducted in 15 °P wort at 15 °C. Prior to bottling, the beers were diluted into the same strength of ABV

	A15 green beer	Sp3 green beer	A15 bottled beer	Sp3 bottled beer
Alcohol by volume (%)	7.0	6.0	4.5	4.5
°Plato	2.5	4.2	1.5	3.1
pH	4.7	4.2	4.8	4.5
Viability (%)	90	39		
Flocculation ^a	+	—		
Fermentation time (h)	310	406		
Diacetyl ($\mu\text{g l}^{-1}$) ^b	34	25	12	20

^aSymbols: — no flocculation, + faint flocculation, + strong flocculation, ++ very strong flocculation

^bThreshold for diacetyl 100–200 $\mu\text{g l}^{-1}$ [39]

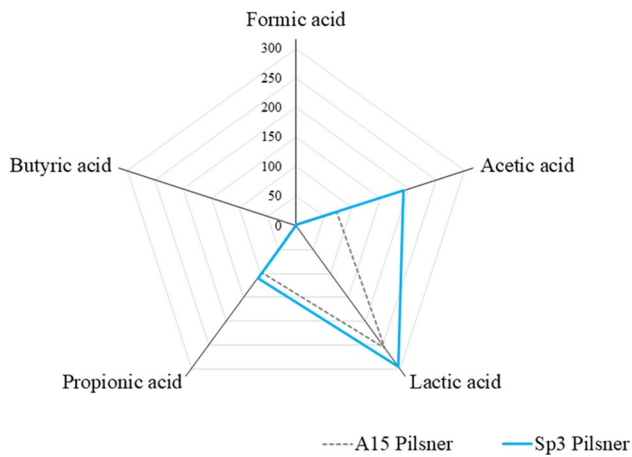


Fig. 7 Organic acids in bottled beers. Beers were fermented in 40-l scale in 15 °P wort at 15 °C. After maturation, the beers were diluted into the same strength (ABV) and bottled

Table 6 Sensory panel results for attributes, which had over 0.5 score at least in one of the evaluated beers

	A15	Sp3
Estery	0.79	1.72
Fruity	0.97	1.61
Hoppy	0	0.52
Phenolic	0	2.54
Fatty acid	1.34	0
Sulphidic	0.54	0
Cooked vegetable	0.57	0
Acidity	0.61	0.82
Sweet	0.68	0.68
Bitter	0.65	1.07
Mouthfeel	0.63	0.59
Carbonation	0.15	0.72
Warming	0.61	0.65
Fullness	0.88	1.06

The attributes were evaluated on a scale from 0, 'nothing' to 5, 'extreme'. Beer flavour profiling attributes introduced by Meilgaard [38] were used. For the complete list, please see the supplementary material S6

just over 10%. Both outcomes are in line with other studies where oak trees were sampled for *Saccharomyces* yeast [4, 5, 26] suggesting that *S. paradoxus* is the dominant species on the oak trees in Northern Europe. Robinson et al. made also a temperature model for predicting the occurrence of *S. paradoxus* and they found that over 95% of *S. paradoxus* isolations were made in areas where the summer temperatures (T_{\max}) were in the range of 20–30 °C. In Finland, oak forests occur naturally in a relatively small area near the coastline between Helsinki and Turku, in which the summer

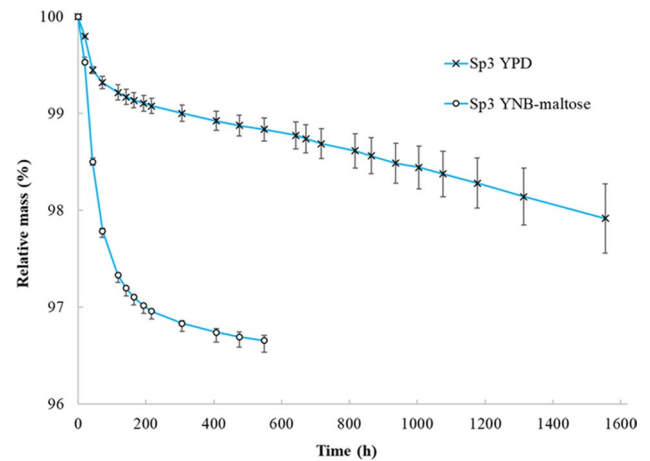


Fig. 8 Relative mass in autoclaved 12 °P wort fermentations with Sp3 strains stored and propagated on/in glucose (Sp3 YPD) and on/in maltose (Sp3 YNB-maltose). Error bars represent the deviation between duplicate

temperatures (T_{\max}) are in the range 21–23 °C [42]. Thus, Finnish oaks appear to be an ideal habitat for *S. paradoxus*.

In the fermentation trials, there was variation between different wild isolates of *S. paradoxus* with respect to both fermentation properties and viabilities, highlighting the importance of careful screening when selecting yeasts for industrial application. At times, variation between replicates was also apparent, as seen with the strain Sp23 (where concentrations of higher alcohols in one replicate were different to other wild isolates, but still comparable to those observed with *S. cariocanus*). Given enough time, most *S. paradoxus* strains demonstrated clear potential for brewery fermentation. The attenuation rates of six out of eight wild strains were around 70% and the best performing strains were able to ferment at temperatures typical for both lager and ale fermentations. Attenuation rates were lower than in fermentations performed with ale and lager yeasts due to the inability of *S. paradoxus* to use maltotriose, a trait which is fairly typical for wild yeasts [30, 41, 43]. The results indicate some similarities and differences compared to *S. eubayanus*, another wild *Saccharomyces* species. That species is similarly unable to use maltotriose, and achieves comparable attenuation levels. In the studies made by Gibson et al. [28] and Magalhães et al. [43], it was seen that in addition to phenolic off-flavours, *S. eubayanus* had also the tendency to produce sulphuric off-flavours during fermentation, which has been related to high expression of genes involved in assimilatory sulfate reduction [44]. This trait was not noticed with *S. paradoxus* strains in this study. Likewise, the high levels of phenyl ethanol that are sometimes observed with *S. eubayanus* were not observed here. *S. paradoxus* strains were also able to tolerate both higher and lower fermentation temperatures, suggesting a more versatile nature compared

to *S. eubayanus*' apparent preference toward lower temperatures [28].

The extended period of inactivity seen in the small-scale fermentations with *S. paradoxus* isolates was also observed previously in fermentations performed with *S. eubayanus* and *S. uvarum* [30, 43]—though the time required for this additional adaptation step seems to vary with species and strain. It is likely that this lag phase is a result of maintenance and propagation in media containing glucose as the only carbon source. The lag phase duration during a glucose-to-maltose shift in *S. cerevisiae* appears to be dependent mainly on the repressive effects of glucose on the respiratory metabolism [45]. With *S. paradoxus*, glucose repression seems to play a strong and a crucial role in the ability of the strains to use maltose. However, by maintaining the cells only on maltose—the additional lag phase seems to be avoidable. In the present study, cropping the yeast from one fermentation, and pitching directly to the next, led to a considerably shorter fermentation time, with a reduction from 26 to 17 days.

Scaling-up fermentation volume (from 100 ml to 40 l) with the best performing *S. paradoxus* strain (Sp3) resulted in reduced production of higher alcohols, but almost doubled the ester concentrations. Hydrostatic pressure could partly explain these results as the higher alcohol concentrations were decreased each time the fermentation volume was increased. With esters, no such correlation was apparent, as the concentrations were at their highest in the 2 l scale. However, a drop in fermentation temperature appeared to increase the production of ethyl acetate and ethyl hexanoate. The concentrations of the latter were steadily above the flavour threshold in fermentations at 15 °C. This may be a consequence of the increased need for unsaturated fatty acids in the cell membrane composition at lower temperature, as the lack of oxygen and the need for unsaturated fatty acids can increase ester production [46]. Although the ester and higher alcohol concentrations decreased from the green beer levels during the maturation and bottling, the fruity and estery notes were still found in *S. paradoxus* beer by the sensory panel and were not masked by the phenolic flavours, which were graded as the most dominant sensory notes.

From a practical perspective, there are a number of things to consider when using wild *S. paradoxus* strains. The viability of isolates was usually low (compared to those of commercial ale and lager strains) and viability of sedimented yeast in the cone was even lower. Therefore, the yeast must be handled carefully if it is to be re-used in subsequent fermentations. The strains also exhibited weak flocculation, leading to high amounts of yeast remaining in suspension that would necessitate removal before packaging. In modern, well-equipped breweries, the weak flocculation can be overcome simply by centrifuging the beer. However, it is likely that improved viability and flocculation could be obtained

through laboratory evolution by circulating sedimented yeast to new batches until the higher viability and flocculation are obtained [47, 48].

Since *S. paradoxus* demonstrated relatively good brewing ability in our fermentation trials, it is reasonable to speculate about its absence in fermentation environments, especially in northern Europe, where it is repeatedly isolated from environmental samples and where there is a strong brewing tradition. In contrast to *S. paradoxus*, *S. cerevisiae* is rarely found in nature and, rather, frequently found in human-associated fermentation environments [25, 49, 50]. Indeed, several separate domestication events have been observed for *S. cerevisiae*, and these are typically associated with signature changes in the genome [49, 51–53]. Strains used for brewing tend to possess the ability to efficiently use maltotriose during fermentation, e.g. through the retention and duplication of genes encoding maltotriose-transporting permeases [51, 52, 54] or a maltotriose-hydrolysing glucoamylase [55]. This ability is rarely found in wild yeast, and is also absent from our tested *S. paradoxus* strains. It is unclear why *S. cerevisiae* has emerged as the dominant *Saccharomyces* yeast in fermentation environments. In addition to being inadvertently spread by human activity, it is tempting to speculate that the *S. cerevisiae* genome may be more susceptible to variation. A small survey of strains from the main populations of *S. cerevisiae* and *S. paradoxus* recently revealed contrasting evolutionary dynamics in regard to the accumulation of balanced and unbalanced rearrangements in these yeasts [2]. These differences may have arisen due to differences in evolutionary history [2], or it may be that the inherent features of the *S. cerevisiae* genome have predisposed this species to domestication. Indeed, lower mutation rates and higher genome stability have been observed in the *S. paradoxus* genome compared to the *S. cerevisiae* genome during laboratory evolution [56]. However, it is possible that prior to the widespread use of pure cultures, *S. paradoxus* may have made a contribution to beer fermentations and some ancient beer may have been fermented by this yeast alone.

To sum up the outcomes of the study, a wild strain of *Saccharomyces paradoxus* isolated from oak bark was used successfully to produce beer at a typical lager beer fermentation temperature of 15 °C at pilot scale (40 l). Pre-adaptation of yeast to maltose-rich conditions appeared to be an important factor for successful fermentation, and re-pitching of yeast cropped from a previous fermentation is recommended as long as viability is carefully monitored. Excluding phenolic notes, the beers lacked any noticeable off-flavours, were considered full-bodied, and generally rated as good as, or better than, the reference beer. The phenolic notes may limit the wider use of the species in brewing, but the growing demand for speciality beers provides a commercial niche for beers made with wild yeasts. Thus, this study supports the

contention that the industrial application of wild *Saccharomyces* yeasts for brewing is feasible as long as the yeast is handled appropriately. Use of *S. paradoxus* and other wild yeasts is expected to help in the crafting of beers with novel quality attributes. Results included here, and from previous studies with *S. eubayanus*, suggest that a comprehensive evaluation of the brewing properties of the remaining members of the *Saccharomyces* genus is warranted.

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Compliance with ethical standards

Conflict of interest After this study was completed, a strain of *Saccharomyces paradoxus* was used for commercial beer production at the Sinebrychhoff Brewery. Otherwise, the authors declare no conflict of interest.

Compliance with ethics requirements Sensory analyses were carried out by a trained panel at the brewery, all of whom gave their prior informed consent to be involved in the trial.

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