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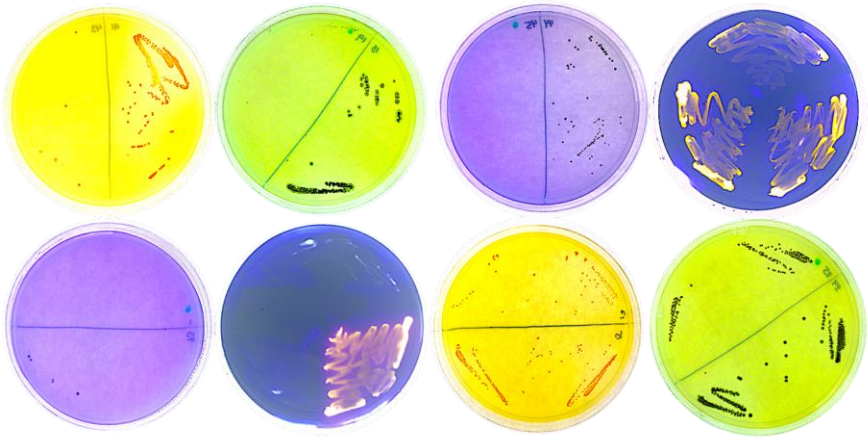

Understanding the emerging beer spoilage yeast *Saccharomyces cerevisiae* var. *diastaticus*

Master's thesis in Biotechnology

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CHALMERS UNIVERSITY OF TECHNOLOGY in collaboration with Craft Labs HB
Gothenburg, Sweden 2019

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Abstract

Saccharomyces cerevisiae var. *diastaticus* has recently become infamous in the brewing industry as a potent beer spoilage organism. This variety of *S. cerevisiae* is characterised by the production of an extracellular glucoamylase, encoded by the *STA* gene family and in particular *STA1*, which allows it to degrade starch and dextrins present in finished beer and thereby prolong the fermentation process. Overfermentation of beer causes excess carbonation, leading to bottle gushing and occasional bursting, as well increased ethanol content, and as such must be avoided by brewers. To further this goal, better understanding of the behaviour of *diastaticus* is required. Notably, the presence of *STA1* in a brewing yeast strain is not always indicative of an overly fermentative phenotype, and indeed there can be large variations between the level of diastatic activity of different *diastaticus* strains. This project investigated the diastatic activity of a wide array of diastatic *S. cerevisiae* strains used in the brewing industry, encompassing all currently identified diastatic brewing yeast types. Assaying of the strain collection for growth on starch revealed distinct diastatic phenotypes, which were then further detailed using selected strains by metabolite profiling using HPLC. *STA1* expression appears to be favoured by normal growth conditions in many strains, but some highly diastatically active were able to utilise starch for growth even in stressed conditions. Sequencing of a fragment of *STA1* from the entire strain collection together with phylogenetic analysis revealed few genetic differences and no genetic basis for variation in diastatic phenotype between *diastaticus* strains. Experimental contamination of finished beer with a strongly diastatically active strain demonstrated the strong spoilage potential of highly diastatically active *S. cerevisiae* var. *diastaticus*, even in refrigerated conditions (8°C). Overall, this project demonstrated the diversity of diastatic phenotypes in *S. cerevisiae* var. *diastaticus* and the importance of taking into account those differences for the brewing industry.

Keywords: *Saccharomyces cerevisiae* var. *diastaticus*, glucoamylase, super-attenuation, beer spoilage, *STA*, *STA1*

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1. Introduction

Saccharomyces cerevisiae var. *diastaticus* yeast strains have the capacity to produce an extracellular glucoamylase encoded by *STA* genes. This enzyme allows *diastaticus* strains to degrade starches and dextrins by releasing glucose units from non-reducing ends (1). This capacity may be considered interesting and desirable in the production of certain beers, namely low-carbohydrate beers, or applied to high gravity brewing (2), as well as in industrial starch fermentation processes. However, *S. cerevisiae* var. *diastaticus* yeasts have also recently become known for their significant spoilage potential in the brewing industry (3). As a beer spoilage yeast, *S. cerevisiae* var. *diastaticus* is capable of fermenting starches and dextrins that most common brewing yeast strains cannot degrade, and thus over-fermenting or, in brewing terms, "super-attenuating" beer. This causes an increase in ethanol and carbon dioxide levels in packaged beer. In particular, increased pressure due to high CO_2 production may cause gushing and bottle bursting (2).

Increased frequency of brewery contamination with *diastaticus* yeast has been observed in recent years, although these do not always become public knowledge. In the United States incidents of *diastaticus* contamination have been accompanied by some high-profile product recalls due to over-carbonated beer gushing from the bottles and cans (2,3), and reports of contaminations and recalls have also increased across Sweden (verbal communication with Craft Labs HB). Most instances of beer contamination by *diastaticus* yeast are secondary contaminations caused by poor hygiene in packaging machinery, namely the filler environment, or biofilm presence in filler pipework (3). Occasional primary contaminations also occur, in which *diastaticus* yeast compete with culture yeast during the fermentation process (3). *S. cerevisiae* var. *diastaticus* strains are often present in the brewing environment as many are used commercially in the brewing industry, in particular for the production of

Belgian, “saison” and “farmhouse” type ales. When used purposefully and according to tried and tested procedures, *diastaticus* strains produce beer with the desired characteristics and are not considered contaminants. These strains are typically the breweries sole ‘house-yeast’, so contamination of other ‘clean’/non-*diastaticus* fermented products is not an issue. However, developments in the craft beer industry in recent years have introduced less experienced brewers to the brewing world, deviation from proper brewing practices, and production of a wide range of styles by most breweries, which presumably resulted in the problematic increase in the occurrence of *diastaticus* yeast contaminations.

1.1. Genetic basis of diastatic phenotypes

S. cerevisiae var. *diastaticus* is characterised by the presence of *STA* genes, which encode extracellular glucoamylases. Glucoamylases are a family of enzymes that remove single glucose molecules from the non-reducing ends of starch and dextrins by hydrolysis of α-1,4 glucosidic bonds (4). The *STA* genes are hypothesized to have originated from the *S. cerevisiae* gene *SGA1*, which codes for the sporulation-specific intracellular glucoamylase Sga1p (5). *SGA1* is homologous to the *STA* genes with exception for the domain at the 5’ end which is necessary for secretion of glucoamylase (5). The *STA* gene family comprises three genes, *STA1*(chr. IV), *STA2* (chr. II), and *STA3* (chr. XIV) (5). The focus of this project will primarily be *STA1*. This gene has been the focus of the majority of studies on *Saccharomyces* extracellular glucoamylase, it is better characterised than *STA2* and *STA3*, and its presence in *S. cerevisiae* strains has become the criteria for categorizing strains as diastatic within the brewing industry. In this project the term “*diastaticus*” will be synonymous with *STA1+*, whereas “diastatic” will refer to the ability to degrade starch and/or dextrins. However, not all *STA1+* yeast strains have been found to present the super-attenuating phenotype (2,6), which indicates that the non-super-attenuating *diastaticus* yeast either do not express the gene, do so

at very low levels, have altered function or localisation of the enzyme, or express the gene only in conditions not encountered in the brewery environment.

The *STA1* open reading frame (ORF) is 2337 bp long, including the stop codon and a 33 bp region upstream of the translation start codon (7,8) . It comprises a hydrophobic leader sequence (HL), threonine- and serine-rich tract (TS), and catalytic domain (CD) (**Fig. 1**) (8,9). The HL sequence acts as a signal peptide (5,8), and the TS region is important for protein secretion (5,10,11).

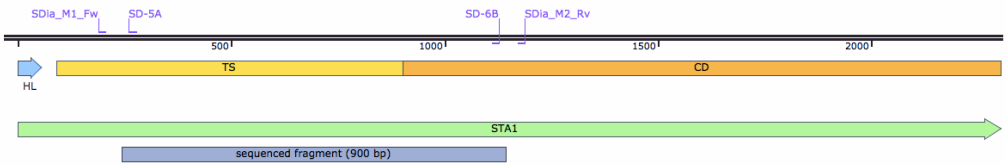

Figure 1. Map of the *STA1* open reading frame (ORF). Numbering of nucleotides begins at the translation start codon. The hydrophobic leader sequence (amino acid 1-21), threonine and serine rich tract (amino acid 22-347), and catalytic domain (amino acid 348-767) are indicated by HL, TS, and CD respectively. The 900 bp sequenced region is indicated, as well as both pairs of primers referred to in this study (see Methods section). The nucleotide sequence can be found with the UniProt Accession No. P04065.

The *STA* genes were found to be repressed in diploids (12) and appear to be carbon catabolite repressed by glucose in some strains (5,13). The promoter sequence of *STA1* is nearly identical to that of the *FLO11* gene, which encodes an extracellular protein involved in invasive growth, pseudo-hyphal development and flocculation (13,14). This suggests that the promoter region of *STA1*, and perhaps also the secretion-specific 5' region of the gene, originated from the *FLO11* promoter (14). Both genes are co-regulated by transcriptional

activators (Flo8, Ms11, Ste12, Tec1) and repressors (13) in a complex environmentally-dependent manner. The promoter region comprises two upstream activation sequences, UAS-1 and UAS-2, which are essential for transcription and play a role in glucose-repression of *STA1* and activation of the gene under non-repressive conditions (7,13).

1.2. Motivation

Expanding our understanding of *diastaticus* yeast to more effectively enable craft and commercial brewers to avoid and manage the risk of diastatic yeast contaminations. Actors within the brewing industry currently have access to limited information concerning the various commercially available strains of *S. cerevisiae* var. *diastaticus.* This is often limited to detection of *diastaticus*, usually by Polymerase Chain Reaction (PCR) testing for presence of the *STA1* gene. As such, the degree to which a given *STA1+* yeast displays diastatic activity is not taken into account, and all *S. cerevisiae* var. *diastaticus* strains are treated as potentially highly diastatic. This project aimed to assess variation in diastatic activity within a large collection of *diastaticus* strains. Differences in sugar metabolism of *diastaticus* yeast were investigated, as well as genetic variation potentially underlying phenotypic differences. *Diastaticus* contamination of finished beer was also reproduced experimentally to evaluate the consequences of various contamination scenarios.

1.3. Scope

A collection of 43 different strains of *Saccharomyces cerevisiae* were used, of which 40 have been confirmed as *STA1+* (using established PCR methods) by either the commercial yeast producer or Craft Labs HB, and 3 non-diastatic control strains (15). Most of these strains are ale strains used both industrially and by homebrewers, but the collection also includes one

lager strain[1], 2 wine strains, one strain isolated as a contaminant from a brewery, and 2 strains isolated from flora in Sweden. All strains as well as their provenance are listed in the Methods section.

Several commercial yeast producers have begun systematically screening their yeast strains for presence of the *STA1* gene and making their results publicly available (to improve customer confidence). Many of these yeasts are similar to each other, as different yeast banks often provide the same or closely related yeast strains. The strain collection used in this project is based on a list compiled by Craft Labs HB containing all currently identified *STA1+* commercial beer strains, excluding redundant strains.

2. Methods

2.1. Strains and media

All strains as well as their provenance are listed in **Table 1**. Original stocks were obtained in lyophilized form or in liquid slurries depending on the producer. Strains were streaked onto YP agar plates containing 20 g L^{-1} maltose to create a strain library. Liquid media used for yeast cultures was either malt extract media composed of 20 g L^{-1} malt extract and 10 g L^{-1} yeast extract or YNB media containing 5 g L^{-1} Yeast Nitrogen Base and 20 g L^{-1} maltose.

Growth assays on solid media were performed with starch agar plates containing 15 g L^{-1} agar, 15 g L^{-1} soluble starch, 6.8 g L^{-1} YNB, supplemented with chloramphenicol at 50 μg mL^{-1} after autoclaving and adjusted to pH 5.2 using 1 M NaOH and 1M HCl, and YPD maltose agar plates

[1] Ales are typically brewed using *Saccharomyces cerevisiae* at 18-24°C, while lagers are usually brewed using *Saccharomyces pastorianus* at fermentation temperatures between 10-13°C However, some strains can be used at both temperature ranges, and some *S. cerevisiae* strains are used as lager yeasts (16, p.106).

as described above. Cultures were incubated at 30°C for both solid and liquid cultures unless otherwise stated.

Table 1. *S. cerevisiae* strains used in this project.

Strain name	**Strain code**	**Company or Provenance**	**Comment**
WLP 073 Artisanal Country Ale	WLP073	White Labs	
WLP 099 Super High Gravity Ale	WLP099	White Labs	
WLP-351 Bavarian Weizen Yeast	WLP351	White Labs	
WLP 545 Belgian Strong Ale	WLP545	White Labs	
WLP 564 Leeuwenhoek saison blend	WLP564	White Labs	Blend of 2 unknown strains
WLP 565 Belgium saison 1	WLP565	White Labs	
WLP 566 Belgium saison 2	WLP566	White Labs	
WLP 570 Belgian Golden Ale	WLP570	White Labs	
WLP 590 French saison ale	WLP590	White Labs	
WLP 644 Saccharomyces 'bruxellensis' trois	WLP644	White Labs	Not *S. bruxellensis*
WLP-707 California Pinot Noir Yeast	WLP707	White Labs	Wine yeast
WLP-740 Merlot Red Wine Yeast	WLP740	White Labs	Wine yeast
WLP-885 Zurich Lager Yeast	WLP885	White Labs	Lager yeast
YeastBay - Dry Belgium Ale 4025	YB-001	Yeast Bay	
YeastBay - Funktown Pale Ale	YB-002	Yeast Bay	
YeastBay - Wallonian Farmhouse	YB-003	Yeast Bay	
WY French saison 3711	WY-3711	WYeast	
WY Belgium saison 3724	WY-3724	WYeast	
WY Canadian Belgian saison	WY-3864	WYeast	
IY-A20 Citrus	IY-A20	Imperial Organic Yeast	
IY-B35 Saison Strain 3	IY-B35	Imperial Organic Yeast	
IY-B56 Rustic	IY-B56	Imperial Organic Yeast	
IY-B64 Napoleon	IY-B64	Imperial Organic Yeast	
OYL-039: Biere de Garde	OYL-039	Omega Yeast	
OYL-040: Belgium dark ale	OYL-040	Omega Yeast	

OYL-042: Belgium saison 2	OYL-042	Omega Yeast	
OYL-055 [No name]	OYL-055	Omega Yeast	
OYL-056 [No name]	OYL-056	Omega Yeast	
OYL-112 Swiss Lager	OYL-112	Omega Yeast	
OYL-200 Tropical IPA	OYL-200	Omega Yeast	
OYL-205 [No name]	OYL-205	Omega Yeast	
OYL-500: Saisonstein's Monster	OYL-500	Omega Yeast	
Lallemand - Belle Saison	L-BS	Lallemand	
MangroveJack - M29 French saison	M29	Mangrove Jack	
Mangrove Jack M31 Belgium triple	M31	Mangrove Jack	Blend (possible contamination)
SafAle-WB-06	WB-06	Fermentis	
SafAle-BE-134	BE-134	Fermentis	
K-yeast - Hisingen farmhouse	K-001	K-yeast	Wild isolate
K-yeast - saison 1	K-002	K-yeast	Wild isolate
K-yeast - saison 2	K-003	K-yeast	Wild isolate
CLY - 24.1	CLY-24.1	Craft Labs	Brewery contaminant
SafAle-US-05	US-05	Fermentis	Non-diastatic control
SafAle-US-04	US-04	Fermentis	Non-diastatic control
Bavarian weizen	M20	Mangrove Jack	Non-diastatic control

2.2. Culturing and screening of growth conditions

2.2.1. Growth profiler

Liquid cultures that were grown in liquid malt media were washed in phosphate-buffered saline solution (PBS) and starved for 72 hrs by incubation in PBS at 25°C. Cells were normalized to Optical Density (OD) 0.02, measured at 600 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany) in the experimental culture media. Each strain was grown in triplicates in 4 different media, all containing 6.8 g L^{-1} YNB and either 20 g L^{-1} soluble starch, 20 g L^{-1} glucose, 10 g L^{-1} soluble starch and 10 g L^{-1} glucose, or

no carbon source. Strains were incubated for 7 days at 30°C in aerobic conditions in a Growth Profiler 960 (Enzyscreen, BV, Netherlands). Averages and standard deviations were calculated from replicate data and media blanks were subtracted for each time point.

2.2.2. Assay for starch utilisation on solid media

Each strain was plated onto starch agar and incubated at 30°C until significant growth was observed in all strains, around 8-11 days for most strains. Yeast biomass was washed off the plate with deionized water and plates were stained with 1.005 g mL^{-1} Gram's iodine solution to visualize if starch had been utilised. This assay was performed once with all 43 strains, and repeated once with strains L-BS, WLP565, IY-B64, BE-134, K-003, WY-3711, WLP885, IY-B56 and US-05, with an incubation of 8 days instead of 11.

2.3. Sequencing of *STA1* and Phylogenetic analysis

2.3.1. Primer design

Primers Sdia_M1_Fw and SDia_M2_R were designed using SnapGene® software (GSL Biotech) according to Eurofins Genomics's specifications (see **Table 2**). The amplified region is 999 bp and comprises the binding sites for the SD-5A and SD-6B primers (17) which are shown in **Table 2**. The primers were checked for specificity using NCBI's BLAST against the entire *S. cerevisiae* genome.

Table 2. Primers used for amplification of a region within *STA1*.

Name	Sequence 5'-3'	%GC	Tm	Source
SD-5A	CAACTACGACTTCTGTCATA	40	50°C	Yamauchi et.al., 1998
SD-6B	GATGGTGACGCAATCACGA	53	54°C	Yamauchi et.al., 1998
SDia_M1_Fw	ATCCAAATACCCTGGCAG	50	54°C	This project
SDia_M2_Rv	CTGTTTATCGTCAACGCG	50	54°C	This project

2.3.2. DNA extraction

DNA was extracted using the following protocol: approx. 50 μL of cells were collected from liquid cultures or plates, washed in MilliQ water and resuspended in 100 μL of 0.5% (w/v) Zymolase in 1 mol L^{-1} sorbitol and 0.1 mol L^{-1} EDTA-Na_2 adjusted to pH 7.5 with HCl to lyse cells. After incubation for 30 min at 37°C, 100 μL of 0.5% SDS in 50 mmol $^{L-1}$ Tris-HCL and 20 mmol L^{-1} EDTA-Na_2 were added and the solution was incubated for 5 min at 65°C. Then 80 μL of 5 mol L^{-1} potassium acetate was added; the solution was cooled at -20°C for 5 min, then centrifuged for 25 min at 10 000 rpm at 4°C. The pellet was discarded and 300 μL isopropanol was added and incubated at room temperature for 5 min. The solution was centrifuged for 5 min at 10 000 rpm at 4°C, and supernatant was discarded. The pellet was resuspended in 300 μL of 70% ethanol, then centrifuged for 5 min, the supernatant was discarded and the pellet air-dried with low shaking at 65°C until all ethanol was evaporated. The DNA was then resuspended in 200 μL MilliQ water and incubated at 50°C shaking at 550 rpm for 1 hr to mix. DNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) and diluted to 15-25 ng μL^{-1}.

2.3.3. Amplification and Sequencing

The 999 bp region of *STA1* was amplified by PCR with primers SDia_M1_Fw and SDia M2-Rv using a MyCycler™ thermal cycler (BIO-RAD, Hercules, USA) and T100™ Thermal Cycler (BIO-RAD, Hercules, USA). One 20 µL PCR reaction contained 0.2 µL of Thermo Fisher Phusion DNA Polymerase, 0.4 µL of 10 mM dNTPs, 4 µL Phusion HF Buffer, 0.5 µL of each primer, and 2 uL of template DNA at a concentration of 15-25 ng μL^{-1}. The annealing temperature was 55°C for strains WLP545, WLP566, WLP644, WLP740, WLP885, YB-001, YB-002, YB-003, WY-3711, WY-3724, IY-B35, IY-B64, OYL-039, OYL-055, OYL-056, OYL-500, K-003, CLY-24.1, M31; 56°C for strains WLP073, WLP351, WLP564, WLP565, WLP570, WLP590, IY-A20, IY-B56, OYL-040, OYL-042, OYL-205, OYL-112, L-BS, M29, WB-06, BE-134; 54°C for strains WLP099, WLP707, WY-3864. Annealing time as well as elongation time was 30 seconds, denaturing time was 15 seconds, and the cycles were repeated 30 times. The amplified DNA was purified using the GeneJET PCR Purification Kit (Thermo Scientific, Waltham, Massachusetts, USA) for all strains except WLP707, WLP740, and OYL042 where DNA was gel extracted with a QIAquick® Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). Control strains US-05, US-04, and M20 were also gel extracted but not successfully purified. Purity and concentration of purified DNA was measured with the NanoDrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA). Samples were sequenced by Eurofins Genomics (Germany) using the provided Sdia_M1_Fw primer.

2.3.4. Data analysis and Phylogenetics

A fragment of the reference sequence for *STA1* from Yamashita et. al., 1985 (8) corresponding to the sequenced region was used for comparison of the sequence data. DNA sequences were trimmed to exclude sections with low quality scores, and aligned to the

reference sequence using MEGA X. Single Nucleotide Polymorphisms (SNPs), insertions and deletions were manually curated by comparison between sequences and observation of sequencing data in Sequence Scanner Software (Applied Biosystems v2.0). Sequences were further trimmed to begin all sequences with a codon and equalize length of sequences.

Phylogenetic analysis was performed using MEGA X according to the protocol by Hall (2013) (18). The cpREV+F model was chosen with amino acid substitution and the reliability of the original tree was estimated by bootstrapping with 2 000 replicates.

2.4. Bottle conditioning

To examine the impact of *diastaticus* yeast on packaged beer, an experiment was performed whereby a fermented beer was inoculated with either a non-diastatic control, a highly diastatic yeast, or a treatment simulating a possible contamination with both non-diastatic and a diastatic yeast. Beer supplemented with 6.25 g L^{-1} glucose was fermented for 6 weeks, at two different industrially relevant temperatures - 8°C and 25°C. Cells were counted under a light microscope using an Improved Neubauer chamber with a depth of 0.1 mm and a chamber size of 0.04 mm^2. Cultures were diluted to appropriate concentrations for counting, and counting results were averaged over 10 counts for each strain. The final concentration of cells was obtained in cells mL^{-1}. Sample treatments were: a mix of L-BS yeast at 10 000 cells mL^{-1} and US-05 yeast at 90 000 cells mL^{-1}, a diastatic treatment with only L-BS at 100 000 cells mL^{-1}, a non-diastatic treatment with only US-05 at 100 000 cells mL^{-1}, and a control group. Samples were prepared in triplicates in 33 cL beer bottles and capped with air-tight caps.

Samples were collected after 3 weeks and 6 weeks from all treatments. Samples were degassed by shaking at 100 rpm for 2 hrs at 15°C and filtered through a 2 µm nylon filter, then frozen at -20°C. The specific gravity of degassed samples was measured with an Oechsle thermo-hydrometer (0.9 – 1.03 g mL^{-1}, calibrated to 20 °C) and adjusted for temperature.

Concentrations of sugars and metabolites in the fermentation media were measured by HPLC using a Dionex™ UltiMate™ 3000 chromatograph (Thermo Scientific, Waltham, Massachusetts, USA) and am Aminex HPX-87H column (30 cm x 4 mm, 2 µm pore size) and quantified using with a refractive index detector. The mobile phase was 5 mM H_2SO_4, flow rate was 0.8 mL min^{-1} at 80°C with an injection volume of 20 µL. Standards of maltotriose, maltose, glucose, fructose, and ethanol were prepared from analytical grade reagents and a 6-point calibration prepared. All calibrations had degrees of correlation greater than 0.99.

2.5. Fermentation and metabolite profiling

Nine strains with different profiles of diastatic activity according to assay results in initial experiments (Section 3.2) were selected for metabolite profiling: L-BS, WLP565, IY-B64, BE-134, K-003, WY-3711, WLP885, IY-B56 and control strain US-05. Fermentations were carried out in triplicates of 60 mL in 100 mL Pyrex bottles with air-locks -filled with 30% glycerol. Fermentations were maintained at 20°C, which is a typical fermentation temperature in most commercial breweries. The fermentation media was sterile wort with an original gravity (OG) of 1.05 g mL^{-1}, which corresponded to an initial fermentable sugar concentration of approximately 36 g carbon L^{-1}. Samples were taken over 10 days for sugar and metabolite profiling by HPLC as described for the bottle conditioning experiment.

2.6. Statistical analysis

Differences between temperatures and strain treatments for final ethanol yields and gravity obtained in the bottle conditioning experiment were tested using GraphPad Prism by two-way ANOVA, and differences between treatments were compared by Tukey's post hoc analysis. Means of metabolite concentrations were examined for statistical differences using Dunn's non-parametric test.. Ethanol yields calculated from the metabolite profiling experiment were compared with a non-parametric Friedmann's test. For all statistical tests the significance threshold (alpha value) was 0.05.

3. Results

3.1. Growth assays

All 43 strains were first assayed for growth under aerobic conditions (using the Enzyscreen Growth Profiler 960 system) on different liquid media in order to determine whether they were able to degrade and utilize starch. **Figure 2** shows the results of cultivation over a 7 day period on media containing either starch, glucose, or no carbon source as controls. Out of the 40 *S. cerevisiae* var. *diastaticus* strains, only 6 showed growth on starch within 150 hrs: WLP590, WY-3711, IY-B64, L-BS, M29, and M31. A fourth media type containing an equal mixture of starch and glucose was also used (**Fig. 3**). In this mixed media, strains either had similar growth to that on glucose (WLP590, WLP644, WLP570, WY-3711, OYL-500, US-04), growth that follows a similar curve to that in glucose media but stops at lower Biomass Unit (BU) (WLP073, WLP707, WLP564, L-BS, IY-A20, IY-B64, YB-003, YB-001, OYL-039, OYL-056, CLY-24.1, K-001, K-003, M20), or growth that appears to continue after a

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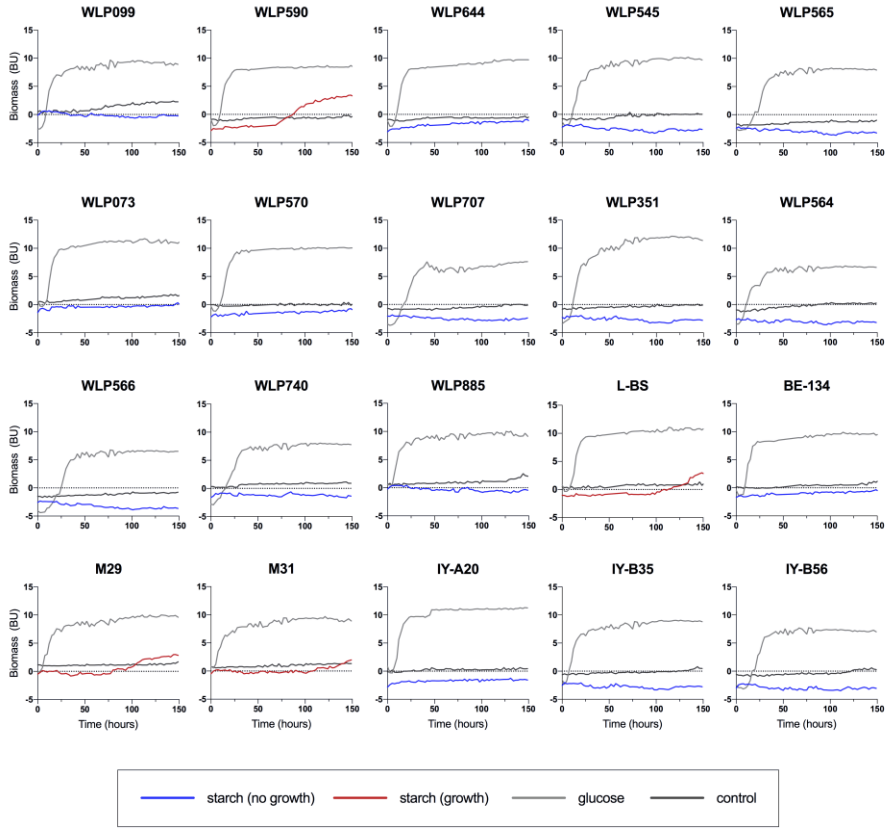
secondary lag phase once growth on glucose has ceased (WLP099, WLP545, WLP565, WLP351, WLP566, WLP740, WLP885, BE-134, M29, M31, IY-B56, WY-3864, OYL-040, OYL-112, OYL-042, OYL-055, US-05). Some strains did not clearly fit into any of these categories (IY-B35, WY-3724, YB-002, OYL-205, WB-06). Control strains (US-04, US-05, M20) each corresponded to a different category.

A



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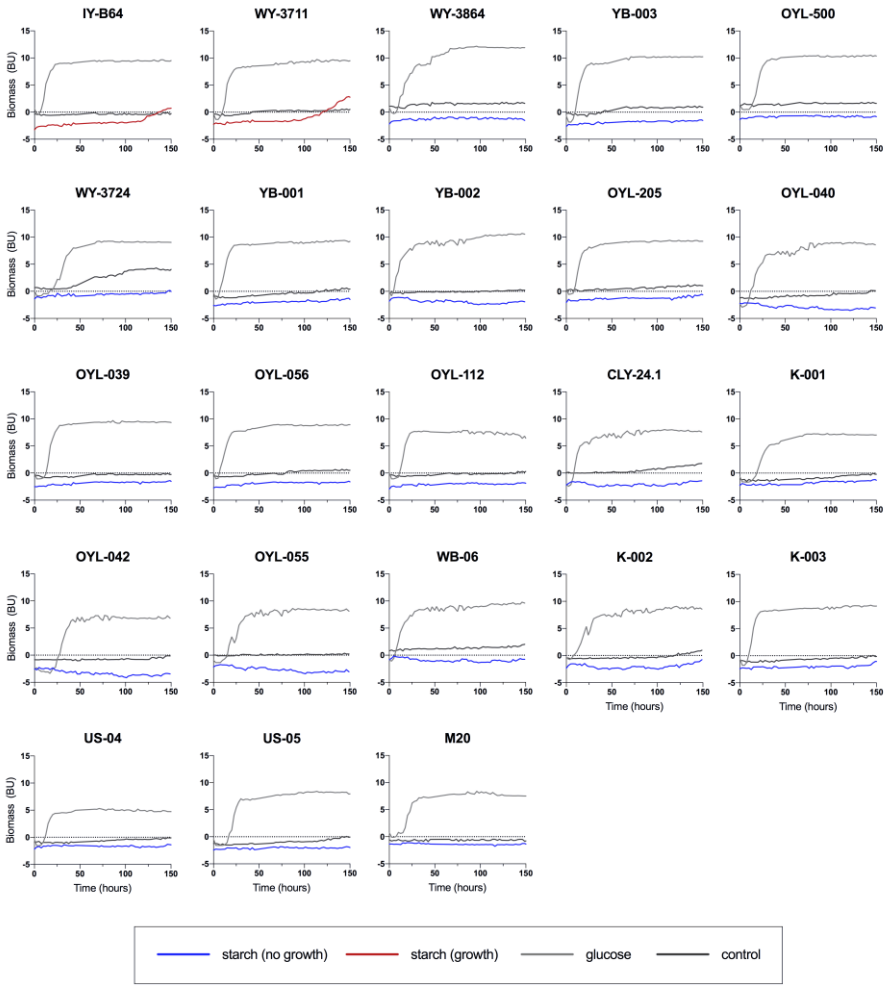
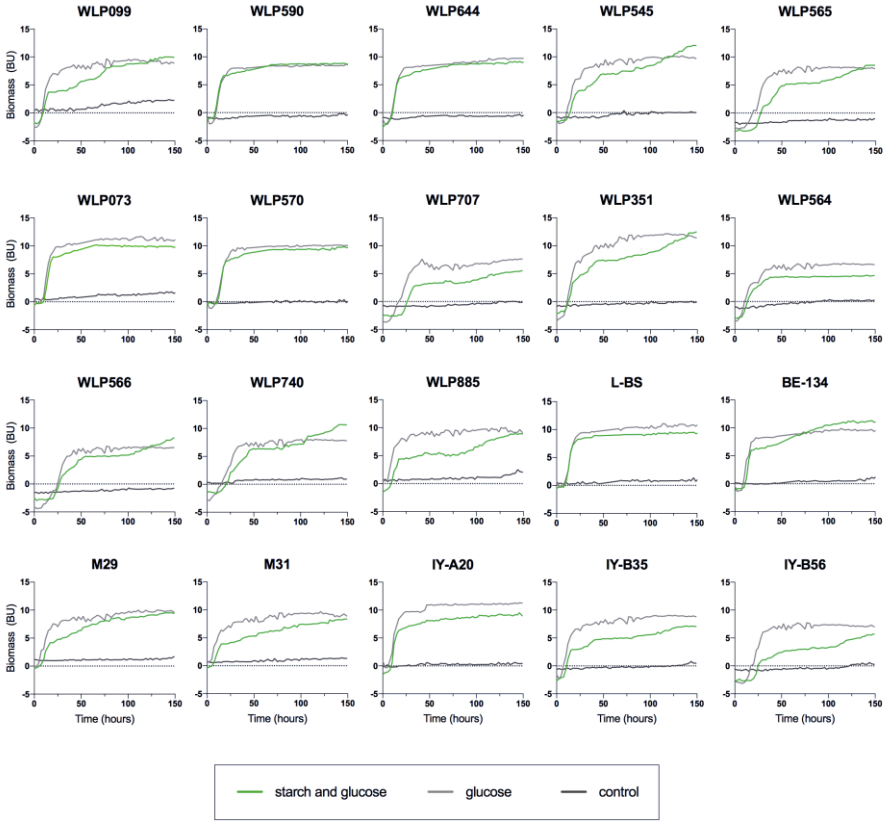


Figure 2. Forty *S. cerevisiae* var. *diastaticus* strains (20 in **A**, 20 in **B**) and three *S. cerevisiae* control strains (**B**)(US-04, US-05, and M20) growing on starch media, glucose media, and a control media without a carbon source. Yeast growth is measured in Biomass Units (BU) which is a value obtained from visual density measurements performed by the Growth Profiler. All data was plotted as averages of triplicate data except for strain WLP740 where a biological replicate was excluded from the dataset.

A


WLP099
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WLP707
WLP351
WLP564
WLP566
WLP740
WLP885
L-BS
BE-134
M29
M31
IY-A20
IY-B35
IY-B56
Biomass (BU)
Time (hours)
starch and glucose
glucose
control

B

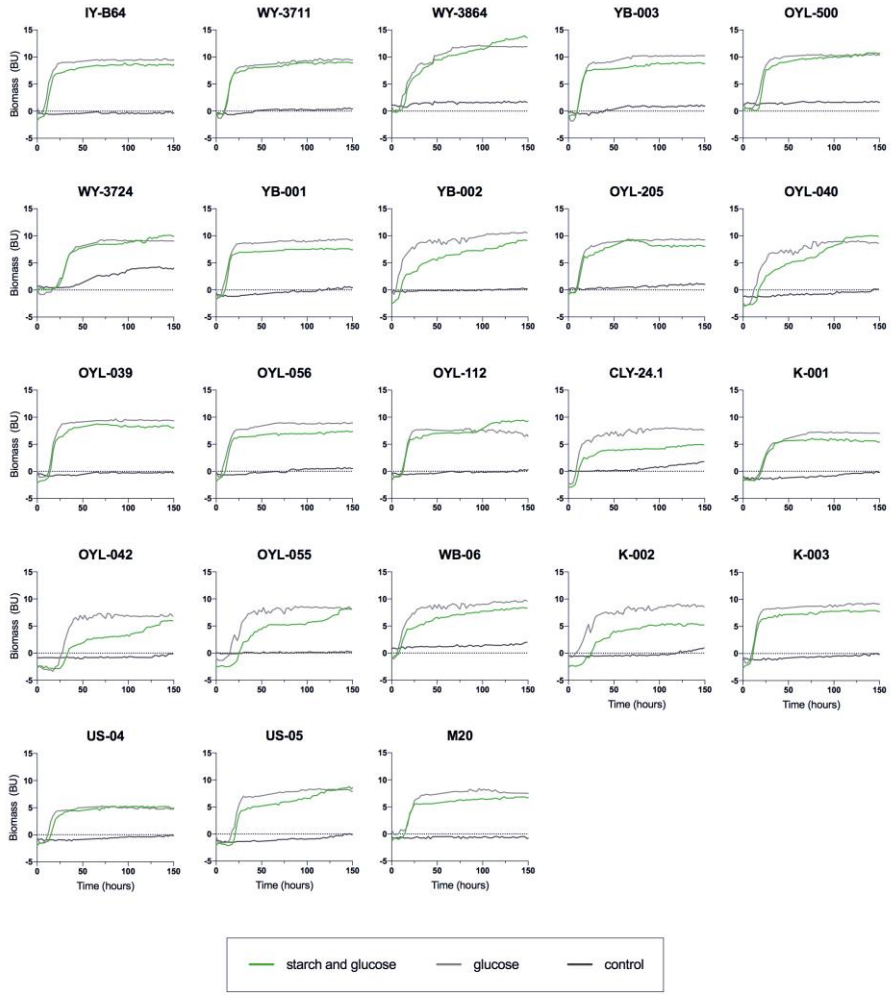

Figure 3. Forty *S. cerevisiae* var. *diastaticus* strains (20 in **A**, 20 in **B**) and *three S. cerevisiae* control strains (**B**) (US-04, US-05, and M20) growing on mixed media containing starch and glucose, glucose media, and a control media lacking a carbon source. Yeast growth is measured in Biomass Units (BU) which is a value calculated from visual density measurements performed by the Growth Profiler. All data was plotted as averages of triplicate data except for strain WLP740 where a biological replicate was excluded from the dataset.

A second assay was performed to detect diastatic activity by measuring starch degradation. All strains were cultured on starch agar plates, which were stained with iodine after 8-11 days of growth. **Figure 4** shows examples of different staining results for a strain that strongly degraded starch (IY-B64), strains that degraded starch locally (IY-B35 and IY-B56), a strain that showed inconclusive results, but may be degrading starch locally (WLP073), and two strains that demonstrated no starch degradation (WLP099 and WLP351). Degradation of starch is presumed to be caused by production and secretion of glucoamylase. The results of this assay for all strains are summarized in **Table 3** alongside the results of the liquid media assay.

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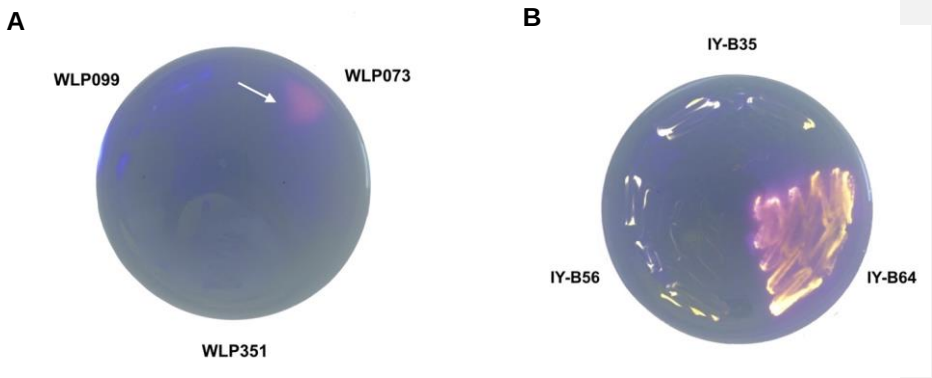

Figure 4. Starch agar plates stained with iodine showing starch degradation by different *Saccharomyces cerevisiae* strains. Areas of lighter colour show where starch was degraded. **A.** Strains WLP099 and WLP351 show no starch use, but WLP073 appears to have a unstained area (indicated by an arrow), which corresponds to less starch in the plate. **B.** Strains IY-B35 and IY-B56 have clear unstained regions, where starch was degraded. Strain IY- B64 appears to have marked starch degradation over the entire area of yeast growth.

Table 3. Results of the starch degradation assay on solid starch media and growth assay on liquid starch media for each of the 43 *Saccharomyces cerevisiae* strains. *S. cerevisiae* strains are grouped according to these results in 5 color-coded categories as follows: **A**: utilised starch on solid media but did not appear to grow on liquid starch media; **B**: *STA1*- control strains; **C**: appeared to utilise starch in neither solid nor liquid media; **D**: results on solid starch media were inconclusive and strains did not appear to grow on liquid starch media; **E**: conclusive starch utilisation in both solid and liquid media. Highlighted strains were assayed twice for starch degradation on solid media with identical results. Evaluation of degradation of starch on solid media and growth on liquid starch media is indicated according to the key at the bottom of the table.

Category	Strain name	Starch degradation on solid media	Growth in liquid starch media
A	WLP564	+	-
	WLP566	+	-
	WLP570	+	-
	WLP707	+	-
	YB-001	+	-
	YB-002	+	-
	IY-B35	+	-
	IY-B56	+	-
	OYL-040	+	-
	OYL-042	+	-
	OYL-055	+	-
	OYL-056	+	-
	OYL-112	+	-
	K-001	+	-
	K-002	++	-
	K-003	++	-
	CLY-24.1	+	-
	BE-134	++	-
	WLP885	++	-
B	**US-05**	-	-
	US-04	-	-
	M20	-	-

Strain name	Starch degradation on solid media	Growth in liquid starch media	Category
WLP099	-	-	C
WLP351	-	-	
WLP545	-	-	
WLP565	-	-	
WLP644	-	-	
YB-003	-	-	
WY-3724	-	-	
WY-3864	-	-	
IY-A20	-	-	
WLP740	-	-	
WB-06	-	-	
OYL-039	(+)	-	D
OYL-205	(+)	-	
OYL-500	(+)	-	
WLP073	(+)	-	
WLP590	++	□	E
WY-3711	++	□	
IY-B64	++	□	
L-BS	++	□	
M29	++	□	
M31	++	□	

Starch degradation on solid media

– no visible starch degradation

(+) inconclusive results

\+ local starch degradation

++ starch degradation beyond colonies (see **Figure 2**)

Growth in liquid starch media

– no observable growth

□ observable growth

3.2. Sequencing and phylogenetics

DNA was extracted from all 43 strains, and a fragment of the *STA1* gene was amplified and sequenced using the Sdia_M1_Fw and SDia_M2_R primers (see **Table 2**). These primers amplify a 999 kb fragment of *STA1*. The non-diastatic control strains were also amplified with these primers and all three yielded a band just below that of the targeted 999 kb fragment, as shown in **Figure 5** for US-05. This amplified fragment was not successfully purified for any of the control strains and therefore not sequenced.

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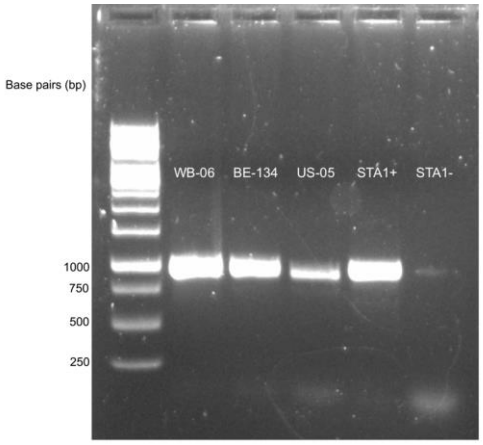

Figure 5. Agarose gel electrophoresis of PCR amplification products for strains WB-06, BE-134, and US-05 using Sdia_M1_Fw and SDia_M2_R primers. DNA fragments were separated by electrophoresis in a gel of 1% agarose in 0.5X TAE buffer.

All strains except those in **Table 4** were confirmed by sequencing of the amplified fragment to possess the *STA1* gene. The sequenced *STA1* fragments are located at the end of the TS region and the beginning of the catalytic domain of the gene, and includes both the SD-5A and SD-6B primers (**Fig. 1**). The obtained sequences were trimmed to 900 bp (**Fig. 1**) aligned and analysed to verify whether the primer-binding sequences for the SD-5A and SD-6B primers contained any mutations and whether the fragments showed any significant function-

altering mutations. This was done for all of the *S. cerevisiae* var. *diastaticus* strain collection with the exception of those listed in **Table 4**. Single nucleotide polymorphisms (SNPs) resulting in codon changes were found in several strains. A total of 14 amino acids were polymorphic within the sequenced fragment for at least one strain. The amino acid substitutions for each strain are listed in **Table 5**. The first 9 substitutions are located within the threonine- and serine-rich tract, and the following 5 occur within the catalytic domain of the enzyme. All sequenced strains were found to have identical primer-binding sequences for SD-5A and only 3 strains were found to have mutations in the binding sequence for SD-6B: WLP564, WLP566, and OYL-055 (see **Table 6**).

Table 4. Criteria for exclusion of *Saccharomyces cerevisiae* var. *diastaticus* strains from sequence and phylogenetic analysis.

Strain	Exclusion criterion
WLP707	Sequenced fragment corresponded to a different loci
WLP099	Failure to amplify *STA1* fragment
WY-3864	Failure to amplify *STA1* fragment
BE-134	Sequence data of insufficient quality for analysis
M31	Blend of different *diastaticus* yeast strains (possible contamination)

Table 5. Amino acid substitutions within the 300 amino acid sequence for all sequenced strains. The codon position is indicated from the beginning of the coding sequence for *STA1*. For each substitution the amino acids are indicated with one-letter codes.

Strain	Position of substitution (codons)						
	229	231	248	273	278	301	311
STA1 partial							
CLY-24.1							
IY-A20				G>C	S>I	C>Y	
IY-B35				G>C	S>I	C>Y	
IY-B56				G>C	S>I	C>Y	
IY-B64							
K-001				G>C	S>I	C>Y	
K-002							
K-003							
L-BS		V>D					
M20							
M29				G>C	S>I	C>Y	
OYL-039			A>T				
OYL-040							S>L
OYL-042							S>L
OYL-055				G>C	S>I	C>Y	
OYL-056			A>T				
OYL-112			A>T				
OYL-205			A>T				
OYL-500					S>I	C>Y	
US-04							
US-05							
WB-06							
WLP073			A>T				
WLP351							
WLP545			A>T				
WLP564				G>C	S>I	C>Y	
WLP565	K>E			G>C	S>I	C>Y	
WLP566				G>C	S>I	C>Y	
WLP570				G>C	S>I	C>Y	
WLP590							
WLP644				G>C	S>I	C>Y	
WLP740				G>C	S>I	C>Y	
WLP885	K>E		A>T				
WY-3711							
WY-3724				G>C	S>I	C>Y	
YB-001			A>T				
YB-002				G>C	S>I	C>Y	
YB-003			A>T				

Strain	Position of substitution (codons)						
	318	346	367	368	369	370	371
STA1 partial	A>V						
CLY-24.1							
IY-A20			L>S				
IY-B35			L>S				
IY-B56			L>S				
IY-B64							
K-001			L>F	P>A	G>W		
K-002							
K-003							
L-BS							
M20							
M29			L>S				
OYL-039		K>E					
OYL-040							
OYL-042							
OYL-055			L>S			V>F	V>L
OYL-056		K>E					
OYL-112		K>E					
OYL-205		K>E					
OYL-500			L>S				
US-04							
US-05							
WB-06							
WLP073		K>E					
WLP351							
WLP545		K>E					
WLP564			L>S			V>S	
WLP565			L>S				
WLP566			L>S			V>S	
WLP570			L>S				
WLP590							
WLP644			L>S				
WLP740			L>S				
WLP885		K>E					
WY-3711							
WY-3724			L>S				
YB-001		K>E					
YB-002			L>S				
YB-003		K>E					

Table 6. Strains with mutation in the primer-binding sequence for SD-6B. The reference sequence, which binds SD-6B as intended is included for comparison. Nucleotides that are not complementary to the primer are highlighted in bold and red. The primer-binding sequences are written 5'-3'.

Strain	Primer-binding sequence (SD-6B)
reference	TCGTGATTGCGTCACCATC
WLP564	**C**CGTGATTGCGTCACCATC
WLP566	**C**CGTGATTGCGTCACCATC
OYL-055	TC**T**TGATTGCGTCACCATC

A phylogenetic tree of all sequenced strains was then constructed in order to observe possible relationships between these strains (**Fig. 6**). The tree was based on the translated amino acid sequences. There did not appear to be sufficient variation between fragments of most strains to infer phylogenetic relationships, as evidenced by the very low bootstrap values of most nodes, showing the high degree of homology in this sequence. Only five strains (the three shown in green and two in blue) with detected polymorphisms cluster with a higher boot strap value.

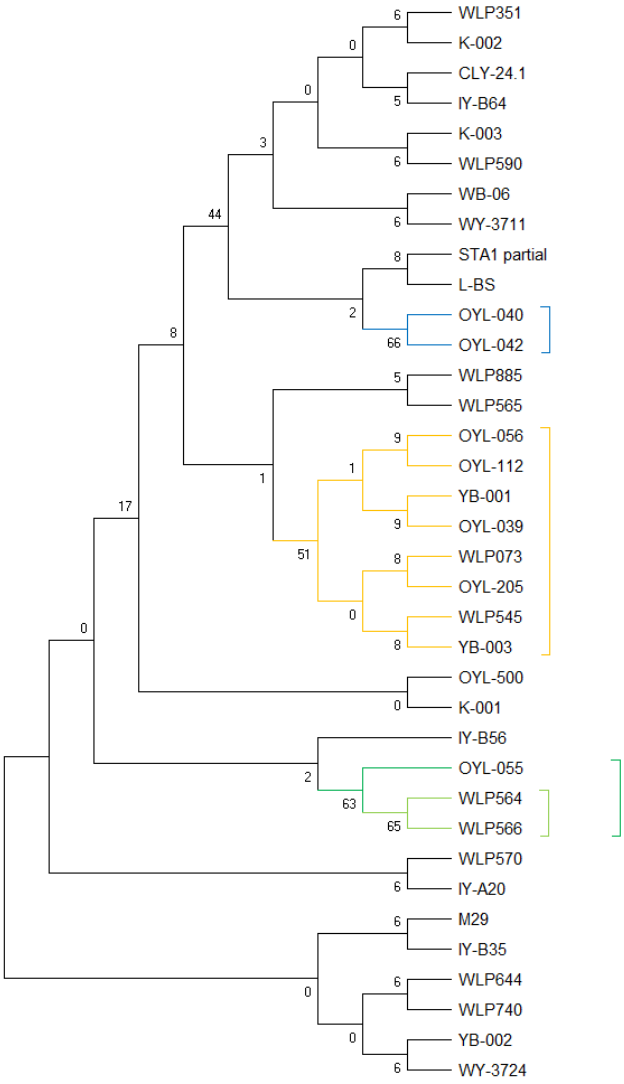

Figure 6. Phylogenetic tree based on 900 bp fragment of *STA1*. The reference sequence "*STA1* partial" is included alongside the sequenced fragments from 35 strains. Bootstrap values are shown at each node and indicate the percentage of reliability of the node. Branches stemming from nodes with >50% reliability are highlighted in colour, all others are not considered reliable.

3.3. Effects of diastatic yeast on packaged beer conditioning

To investigate the impact of a contamination of diastatic yeast in a bottled beer, three different experimental treatments with different yeast were compared in a 6-week ‘bottle conditioning’ experiment using fully fermented beer spiked with a small amount of glucose as fermentation media. This practice is typical for some beers, where the beers are ‘bottle conditioned’ by the addition of extra sugar post fermentation and yeast added to produce a carbonated beverage. This is especially common in small craft breweries and breweries with a focus on producing traditional styles. The first treatment was a mix of a strain with pronounced diastatic activity, L-BS, and a non-diastatic yeast, US-05. L-BS was found to be among the most diastatically active strains identified in the preliminary screening. L-BS made up 10% of the total yeast inoculate and US-05 90%, which imitated a possible contamination, albeit at quite a high level, during the production or packaging process. The second and third treatments were inoculated with either 100% L-BS or 100% US-05, respectively, and bottles with no yeast added were used as controls. All bottles had the same yeast cell count at inoculation. Each of these treatments were incubated both at 25° and at 8°C.

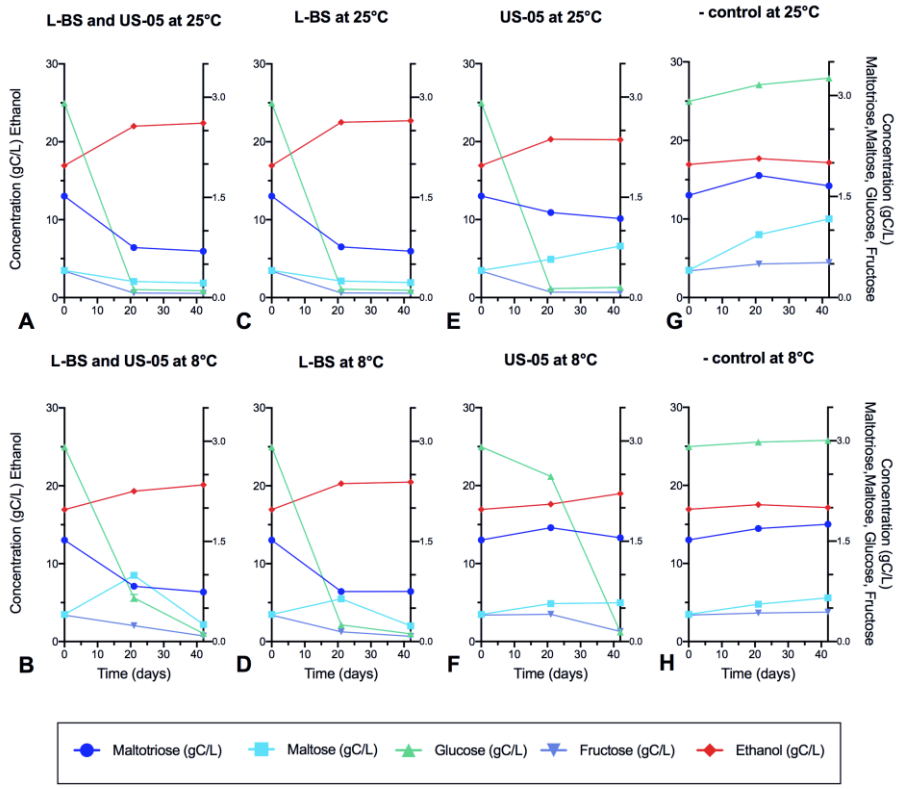

Figure 7. Concentrations of fermentable sugars and ethanol in g Carbon L^{-1} measured over the duration of a 6-week fermentation for different *S. cerevisiae* strain treatments and bottle conditioning temperatures. Four different treatments are shown, consisting of a mix of 10% L-BS and 90% US-05 (**A** and **B**), L-BS alone (**C** and **D**), US-05 alone (**E** and **F**), and a control with no yeast (**G** and **H**). Each treatment was incubated at 25°C (**A, C, E,** and **G**) and at 8°C (**B, D, F,** and **H**). Data points represent the mean value of triplicate bottles sampled after 3 weeks and 6 weeks, analysed in triplicate via HPLC. Error bars represent ± 1 standard deviation.

Quantification of fermentable sugars (maltotriose, maltose, glucose and fructose) and ethanol in each of these treatments held at both temperature was performed after 3 and 6 weeks of conditioning by sacrificing 3 of the 6 bottles for each treatment (**Fig. 7**).The mixed L-BS and US-05 treatment and L-BS treatments held at 25°C (**Fig. 7 A** & **C**) have the same profile for

all sugars and ethanol concentration ($p>0.05$ for all compounds). The US-05 treatment at 25°C (**Fig. 7 E**) had similar curves for glucose and fructose utilisation as the mixed and L-BS treatments at 25°C, but lower maltotriose consumption and lower ethanol production, as well as an increase in maltose concentration compared to the mixed and L-BS treatments (**Fig. 7 A** & **C**). At 8°C the mixed treatment displayed markedly slower glucose consumption than L-BS (**Fig. 7 B** & **D**). Both of these treatments had an initial increase in maltose concentration followed by a decline, with the maltose concentration being lower after 6 weeks than the starting concentration. The US-05 treatment at 8°C consumes glucose and fructose much slower than any other sample, and had an overall increase in maltose concentration, and a slight initial increase in maltotriose that then decreased to approximately the original concentration (**Fig. 7 F**). The negative controls, which did not contain yeast, showed no production of ethanol at either temperature (**Fig. 7 G** & **H**). However, at 25°C glucose and maltose concentrations increased in the control treatment, and maltotriose initially increased and decreased again later on (**Fig. 7 G**). Similarly, the control at 8°C had a very slight increase in maltose and maltotriose concentrations (**Fig. 7 H**).

The treatments held at 25°C had greater final ethanol yields (g C ethanol / g C sugar, **Fig. 8 A**) than those incubated at 8°C ($p<0.0001$). The L-BS treatment had a significantly higher ethanol yield at both temperatures ($p<0.05$ compared to the mixed treatment at both 25°C and 8°C; $p<0.0001$ compared to US-05 at 25°C and $p<0.001$compared to US-05 at 8°C). At 25°C the mixed treatment has a significantly higher yield than US-05 ($p<0.0001$). All three treatments result in significantly higher ethanol yields at 25°C than 8°C ($p<0.0001$).

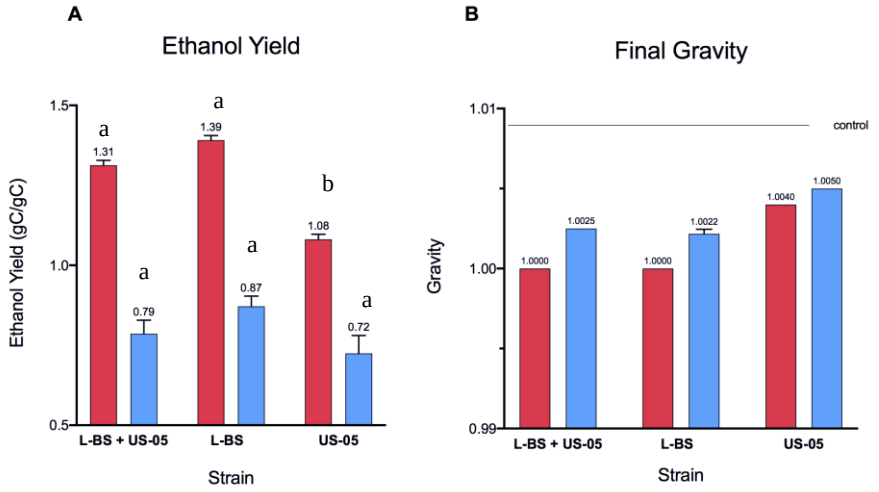

Figure 8. Ethanol yields (A) and final specific gravity (B) after 6 weeks of bottle conditioning for three different treatments at two different temperatures. Each sample treatment was incubated at 25°C (red) and 8°C (blue). **A.** The mean ethanol yield after 6 weeks is shown in g Carbon (ethanol) / g Carbon (total fermentable sugars) for each sample treatment as well as error bars for ±1 standard deviations. **B.** The gravity of the sterile control treatment for both temperatures (1.00917) is indicated by a horizontal line. Statistically significant differences were tested for using a two-way ANOVA and Tukey's post hoc test (for **A** & **B**).

The specific gravity (g mL^{-1}) of the beers conditioned at 25°C was lower than at 8°C for all three treatments at both sampling points ($p<0.0001$; **Fig. 8 B**). A lower final gravity indicates a more fully fermented sample, with less sugars remaining and typically correspondingly greater ethanol content. The mixed treatment has the same final gravity as L-BSat both 25°C ($p>0.05$) and 8°C ($p>0.05$). The US-05 treatment has higher gravity than both of the other treatments at both temperatures ($p<0.0001$), with values that are typical for a finished 'bottle conditioned' beer. All treatments at both temperatures have significantly lower gravity than the control, which remained unchanged during the experiment ($p<0.0001$).

3.4. Fermentation and metabolite profiling

Eight *S. cerevisiae* var. *diastaticus* strains and one non-diastatic control strain (US-05) were selected to be compared in greater detail during a 10-day fermentation at 20°C using wort as the media. Concentrations of fermentable sugars (maltotriose, maltose, glucose and fructose) and ethanol were measured by HPLC at eight time points during the fermentation for each strain (**Fig. 9**). WLP565 and the control strain (US-05) had similar profiles for all sugars and ethanol, and IY-B56 was comparable to these two strains except for the slower maltotriose consumption and higher final ethanol concentration. For the strains: IY-B64, BE-134, and K-003, the glucose concentration decreased rapidly during the first 2 days and then decreased more slowly up to day 10. For L-BS, WY-3711 and WLP885, the glucose concentration increased slightly at 3-4 days before decreasing again. The maltotriose concentration decreased sharply (more than 2.5 g C L^{-1} consumed by day 2) for L-BS, WLP885, K-003, IY-B64, and WY-3711 (**Fig. 9 A, C, E, G & H**), but was much slower for the other strains (less than 1.5 g C L^{-1} consumed by day 2) (**Fig. 9 B, D, F, I**).

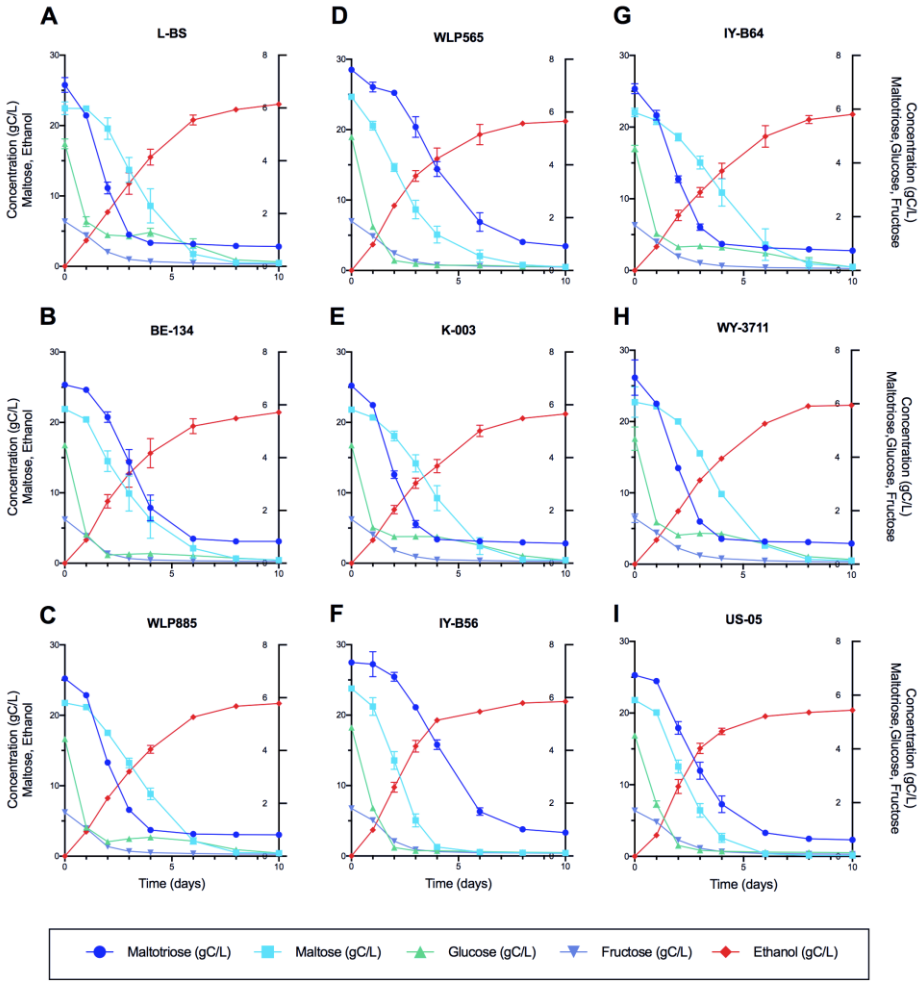

Figure 9. Concentrations of fermentable sugars and ethanol in g carbon L^{-1} for 8 *Saccharomyces cerevisiae* var. *diastaticus* strains and a control strain over 10 days. Strains in graphs **A-H** are *STA1+*, the non-diastatic control strain is US-05 (**I**). Error bars indicate ±1 standard deviation.

The ethanol yield from total fermentable sugars (g carbon (ethanol) / g carbon (total fermentable sugars)) for these strains was measured over the duration of the fermentation

(**Fig. 10 A & B**). Final yields are compared in **Fig. 10 C**. The four strains with the highest yields (WLP885, BE-134, K-003, L-BS) each had significantly higher yields than WLP565 ($p<0.05$ for WLP885, BE-134, K-003, L-BS). WLP885 had a higher yield than US-05, IY-B56 ($p<0.05$), and IY-B64 ($p<0.05$). BE-134 had a higher yield than IY-B64 ($p<0.05$) and IY-B56 ($p<0.05$), and K-003 had a higher yield than IY-B64 ($p<0.05$).

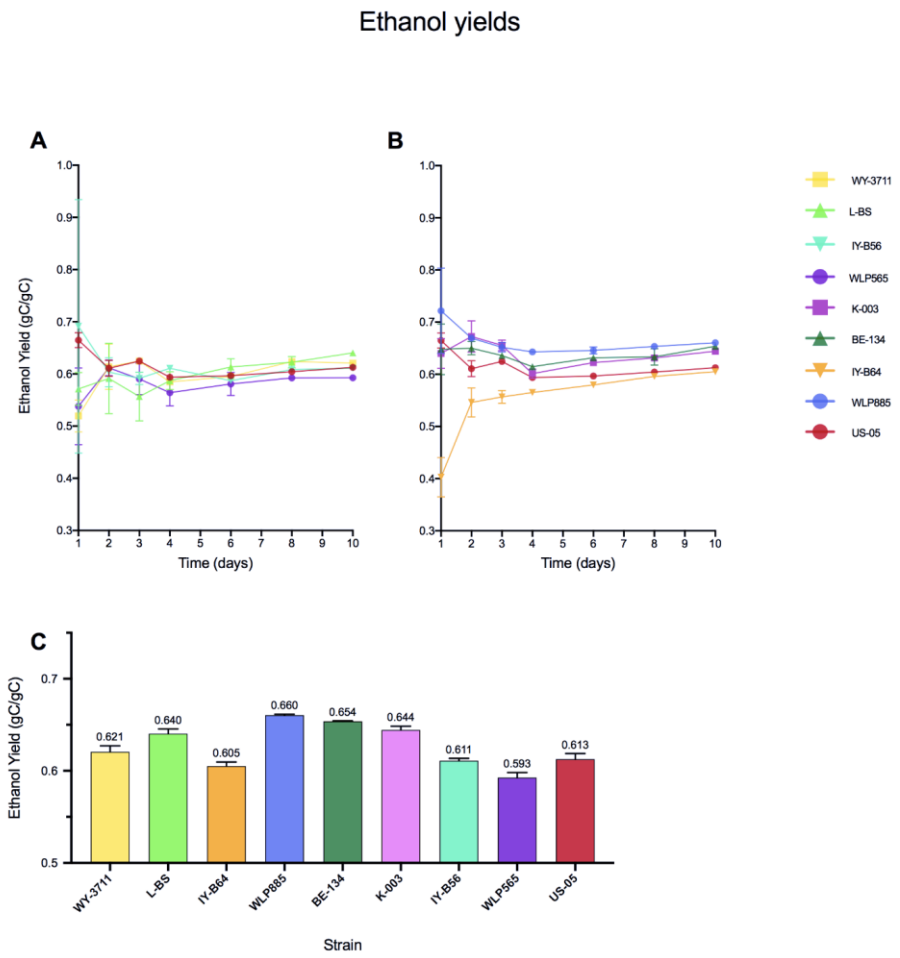

Figure 10. Ethanol yields for nine *S. cerevisiae* strains. A & B. Ethanol yields over a ten-day fermentation period. The mean ethanol yield at each time point is shown in g carbon (ethanol) / g carbon (total fermentable

sugars) and error bars indicate ±1 standard deviations. **A.** Strains WY-3711, L-BS, IY-B56, WLP565, and US-05 are plotted from day 1 to 10. **B.** Strains K-003, BE-134, IY-B64, WLP885, and US-05 are plotted from day 1 to 10. **C.** Final ethanol yields at the end of the fermentation**.** Error bars represent ±1 standard deviation. Statistically significant differences were tested for using a one-way ANOVA and Friedman's post hoc test.

4. Discussion

4.1. Evaluation of diastatic activity and classification of strains

The two existing studies assaying diastatic activity of several *S. cerevisiae* var. *diastaticus* strains have both observed variation in diastatic ability (2,6). Meier-Dörnberg et. al (2018) and Krogerus et. al (2019) both assayed strains for growth on starch agar media, (18 *STA1+* beer spoilage strains and 15 *STA1+* strains, respectively) from both commercial yeast producers and research culture collections(2,6). This project used 40 *STA1+* strains, including 34 commercially available beer strains, 2 wine strains, 3 wild isolates and 1 brewery contaminant. While accounting for probable repetition of some strains (commercial producers are known to change names of strains already on the market), the commercial beer strains used in this project cover all of the currently identified commercially available *STA1+* beer strains. This project therefore gives a more comprehensive overview of the variation in diastatic potential in beer yeast.

In previous studies, diastatic *S. cerevisiae* var. *diastaticus* strains were assayed for growth on starch rather than starch degradation (2,6). Krogerus et. al (2019) performed starch growth assays of *S. cerevisiae* var. *diastaticus* strains under anaerobic conditions after washing cells to remove all fermentable sugars from the culture media. Meier-Dörnberg et. al (2018) starved cells to deplete glycogen stores before incubation on starch media, and assayed for both aerobic and anaerobic growth. Aerobic and anaerobic conditions favour different

metabolic pathways in *S. cerevisiae* (19), and sugar starvation of *S. cerevisiae* also modifies metabolism (20), which may have an impact on production and secretion of glucoamylase. In contrast, the starch degradation assay in this project was performed without washing or starving cells, under aerobic conditions. All strains grew on the starch media, indicating that intracellular glycogen stores and residual fermentable sugars from the inoculate media were substantial enough to enable growth. Certain strains (WLP566 and WLP570) were also shown to degrade starch under these conditions as visualised by iodine staining (**Table 3),** whereas Krogerus et. al (2019) did not observe any growth for these same strains (6). This difference may be due to differences in aerobic and anaerobic production of extracellular glucoamylase, or to metabolic changes in stationary versus growing *S. cerevisiae.* On the other hand, strain-dependent glucose repression of *STA1* is well documented (5,21), and may have led to reduced glucoamylase production for certain strains compared to studies where cells were washed before assaying.

The 40 *STA1+* strains used in this study were also assayed for growth on liquid starch media (**Fig. 2**). For this assay, the cells were first starved before incubation in aerobic conditions. All of the strains that grew under these conditions were also found to be highly diastatically active shown by the solid starch degradation assay. These results appear to confirm that *STA1+* strains with low diastatic activity (on solid media) are also not able to grow aerobically on starch if previously starved, but display diastatic activity when their growth is supported by fermentable sugars (**Table 3)**. Variation in diastatic activity may also reflect differences in the amounts of glucoamylase produced, localisation of the enzyme (complete versus partial secretion, or eventually localisation to the membrane), or enzymatic functionality. The results of these assays may be cautiously generalised to anaerobic conditions as well, as results of preliminary assays performed by Meier-Dörnberg et. al

(2018) indicated that assays of *S. cerevisiae* var. *diastaticus* on starch media produce identical results in aerobic and anaerobic conditions, provided that cells are washed before inoculation and no ethanol is present in the culture media (2).

No conclusions regarding diastatic phenotype can be drawn from the liquid media assay for growth on mixed glucose and starch media (**Fig. 3**). Three distinct patterns emerged for growth on this mixed media: growth curves identical to growth on glucose, curves similar in shape to growth on glucose but stopping at a lower amount biomass, and growth that stabilizes as for growth on glucose but continues to increase afterwards. For some strains growth curves corresponded to none of these three trends. The growth curves of the three *STA1-* control strains each corresponded to a different pattern, which indicates that growth of *S. cerevisiae* on mixed starch and glucose media is not determined solely by diastatic activity.

The measurement of fermentable sugars and ethanol during fermentation of the eight more closely studied *STA1+* strains highlighted more subtle differences in diastatic phenotype than the growth assays (**Fig. 9**). Cultures of strains L-BS, WY-3711 and WLP885 displayed an increase in glucose concentration after the initial drop, which indicates that glucose was released in media faster than it was consumed, and is indicative of high glucoamylase activity. Strains IY-B64, BE-134, and K-003 appear to have intermediate glucoamylase activity, as glucose was released at a rate similar to that of consumption. These fermentation profiles correspond well to the categories formed on the basis of the liquid starch growth assay and solid starch degradation assays. Strains IY-B56 and WLP565 have similar profiles showing low diastatic activity when grown on wort media, but when taking into account the solid starch degradation assays (**Table 3**) IY-B56 appears to degrade starch to some extent whereas no glucoamylase activity was observed for WLP565. Together, the results show that

L-BS, WY-3711, and WLP885 have high diastatic activity, IY-B64, BE-134, and K-003 have intermediate diastatic activity, IY-B56 has low activity and WLP565 has the lowest of the tested strains.

4.2. Primers and sequencing

Testing for the presence of the *STA1* gene in *S. cerevisiae* strains is usually done by PCR. The primer pair most often used by commercial yeast producers and quality control labs are SD-5A and SD-6A and all of the strains selected for this study have previously been confirmed as *STA1+* using these primers. Use of the SD-5A and SD-6B primers present a slight technical issue, in that they have a difference in annealing temperatures which makes it difficult to obtain a clear band for the *STA1* fragment. The primers designed in this study did not however succeed in improving identification of *STA1* by PCR, as amplification using these primers produced secondary bands just below the *STA1 fragment* for some strains, including *S. cerevisiae* that were previously confirmed to be non-diastatic. Certain strains identified by their producer as *STA1+* (using the original primer set) could not be successfully amplified using the primers designed in this study (**Table 4**), but further tests are required to verify whether this was due to a failure of the primers or to other factors, such as DNA quality. For all other strains, sequencing was able to confirm presence of *STA1* (although sequence data for strains BE-134 and M31 was of too low quality for further analysis). Sequencing of the fragment amplified from strain WLP707 revealed that the primers had amplified a fragment in an entirely different region of the *S. cerevisiae* genome, so it remains to be proven in this study that this strain is indeed *STA1+* and have to rely on its identification as such by the commercial producer. Intriguingly, PCR of the non-diastatic control strains produced a band slightly below 1000 bp. This band may correspond to the same sequence as that of secondary bands obtained in other strains for trial amplifications

(data not shown), but determination of its origin would have required significant scale-up and purification work as the band was so faint and as such was left out of the scope of this study.

Although the main aim for this study was not to develop new primers for identification of *STA1+* strains, but more to enable phylogenetic analysis, these results serve to highlight the difficulty in developing effective primers for this gene. Furthermore, this study has shown that not all strains which were confirmed by sequencing to be *STA1+* were in fact diastatically active. This indicates the need for more refined detection methods for *S. cerevisiae* var. *diastaticus.*

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YES

4.3. Genetic basis for variability?

Low or unobservable diastatic activity in several commercial *S. cerevisiae* var. *diastaticus* beer strains was recently linked to a 1162 bp deletion of the promoter region of *STA1* by Krogerus et. al (2019)(6). An earlier study of the *STA1* promoter found that the upstream activation sequences UAS-1 and UAS-2 are required for production of glucoamylase (7). One of these sequences sufficed for production of significant amounts of glucoamylase, but when both were deleted, no enzyme was produced. The 1162 bp deletion by Krogerus et. al (2019) contains the UAS-2 region but not UAS-1. Deletions of similar regions of the promoter, containing UAS-2 and a downstream region, were shown by Shima et. al (1989) to correspond to 64-100% of control glucoamylase activity (7). Strains with the 1162 bp promoter deletion were able to degrade dextrins to varying degrees, but do not grow on starch media or in fully-attenuated beer (6). This further supports the conclusion that strains with low diastatic potential can be diastatically active when fermentable sugars are available to support growth. The 1162 bp promoter deletion was found in four strains used in this study, WLP565, WLP566, WLP570, and WLP644, all of which exhibited intermediate diastatic

activity versus for instance highly diastatically active strains L-BS or WY-3711 (**Table 3**). Strain WY-3711 has an intact promoter region (6), and displays correspondingly high diastatic activity according to both this study and Krogerus et. al (2019). As mentioned above, strains WLP566 and WLP570 displayed diastatic activity despite featuring this deletion in the *STA1* promoter. Altogether, it appears that although this deletion reduces diastatic potential, it does not eliminate it entirely, and variation in diastatic activity occurs even between strains with this deletion. There are still more than a dozen strains which are *STA1+* and have no measurable diastatic activity (under conditions thus far tested) for which no genetic or regulatory basis for this phenotype have been found. Such subtle variations in diastatic phenotype may be caused by mutations in any of the several genes involved in regulation of *STA1*, or by as-yet undiscovered mutations in either the promoter region or open reading frame of *STA1* itself.

The 900 bp region of the *STA1* codon sequenced (using primers Sdia_M1_Fw and Sdia_M2_Rv) in this study did not reveal any mutations that could explain phenotypic differences within this fragment. Nine of the fourteen amino acid substitutions found were located in the threonine- and serine-rich domain, which is reportedly responsible together with the leader peptide sequence for secretion of extracellular glucoamylase (see **Fig. 1** for structure of the *STA1* gene) (10,11). The remaining five substitutions were located in the catalytic domain, but none of the asparagine residues that are proposed by Adam et. al (2004) as potential N-glycosylation sites are modified in any of the sequenced strains (11). Phylogenetic analysis of the translated amino acid sequence did not reveal any clusters corresponding to the established phenotypic groups (**Table 3**), and there is no observable correlation between amino acid substitutions and phenotype (**Table 5**), which suggests that these mutations are not responsible for the observed variation in diastatic potential. However,

differences in diastatic activity caused by any of these substitutions cannot be entirely ruled out without further research into Sta1 glucoamylase localisation and structure.

In conclusion, stark differences in diastatic potential appear to be explained by the 1162 bp promoter deletion for at least some strains as found by Krogerus et. al (2019) (6), but not by the 900 bp region targeted in this study. Further research is needed to verify whether all *STA1+* strains displaying low diastatic potential also have this deletion and to rule out the existence of any other functionally significant polymorphisms within the *STA1* gene and promoter region.

4.4. Beer contamination by *S. cerevisiae* var. *diastaticus*

Having identified strains that clearly present a diastatic phenotype, one of these was selected to investigate the impact of the activity of *STA1* glucoamylase in packaged beer, where the majority of contaminations have previously been found to originate (3). The objective was to compare fermentation profiles and attenuation (change in specific gravity of beer due to the conversion of fermentable sugars to alcohol (16, p.87)) of contaminated and non-contaminated beer at temperatures corresponding to refrigerated (8°C) or non-refrigerated (25°C) storage in an industrial setting.

It is worth noting that there were slight changes in the carbohydrate profile of the control beers, with an increase in fermentable sugars after 3 and 6 weeks in these bottles. This could possibly be attributed to some oxidative breakdown of starch in these beers. The bottles were not purged of air before filling as would be typical in the production environment, thus a high amount of dissolved oxygen is expected to be present in these bottles upon packaging

The results showed that the “contaminated” bottles (mixed diastatic and non-diastatic strains) had much closer fermentation profiles to that of pure diastatic yeast than non-diastatic yeast (**Fig. 7** & **8**). The “contaminated” and diastatic samples behaved almost identically at 25°C, but some differences between these inoculates were noticeable at 8°C. In particular, it appears that L-BS is more active at 8°C than US-05, as the former consumes sugar and produces ethanol much faster than the later, and the “contaminated” sample containing both strains shows markedly slower glucose consumption than the diastatic sample. An inoculum of 10% of the total cell number is quite high to simulate a contamination and a further study investigating different levels of contamination would be warranted to truly reveal the potential consequences of contamination in a brewing environment. Anecdotally, we found that beers inoculated with L-BS were more carbonated than those with just US-05, and a future study measuring this difference and the pressure levels achieved would be useful as this poses a significant health and safety issue.

The two different temperatures were investigated to ascertain whether cold storage of the beer is enough to prevent a diastatic contamination from spoiling a batch of beer. Typical temperatures for a beer storage warehouse are 8-10°C (communication with local breweries via Craft Labs HB), while 25°C simulates a warm summer with non-refrigerated storage and a possible worst case scenario. It was shown that even at 8°C, the treatments containing L-BS were still ‘spoiled’ as there was a significant degree of fermentation beyond that of US-05, indicative of the utilization of the unfermentable dextrins remaining from the wort. Prioritizing contamination prevention is hence paramount to avoid beer spoilage by highly diastatic yeast.

5. Conclusions

Although the over-carbonation and super-attenuation resulting from contamination of a product by a highly diastatic strain are undesirable for brewers, not all *STA1+* strains pose a risk. Diastatic phenotypes of *S. cerevisiae* strains confirmed by sequencing as *STA1+* ranged between (a) diastatically active both when growing with starch as sole carbon source and when growing on media containing growth-sustaining fermentable sugars, (b) diastatically active only under normal growth conditions supported by fermentable sugars, and (c) diastatically inactive under all tested conditions. The strains with low to undetectable amounts of diastatic activity may in fact be diastatically active under different fermentation conditions to those tested here (pH, alcohol content, glucose concentration, temperature), but other studies have found similar trends (2,6). Several lines of evidence indicate that certain *STA1+* strains have low to negative diastatic activity when sugar limitation restricts growth (as would be the case in most finished beers or media devoid of fermentable sugars), but are diastatically active when growth is supported by the availability of fermentable sugars. In strains where glucoamylase production is glucose repressed, it is possible that glucose concentrations in the fermentation media must be low for repression to be limited at the same time as other fermentable sugars support growth of yeast cells, in order for these strains to exhibit diastatic activity. New detection methods for breweries should account for the high variability of diastatic phenotype among *STA1+* strains.

The results of the bottle conditioning experiment investigating the potential for spoilage of bottled beer by diastatically active *STA1+* yeast highlight the importance of the prevention of contamination. Samples mimicking contamination with small inoculates of the highly diastatically active *diastaticus* strain L-BS added to non-diastatic US-05 were over-attenuated at 25°C and to a slightly lesser extent at 8°C as well. L-BS proved more metabolically active

at 8°C than non-diastatic US-05. Refrigeration does not appear to suffice to prevent spoilage of beer in case of contamination with diastatically active *S. cerevisiae* var. *diastaticus.*

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