Manuscript - with author details

Improved Functional Assays and Risk Assessment for *STA1+* Strains of *Saccharomyces cerevisiae*

Laura T. Burns[1], Christine D. Sislak[2], Nathan L. Gibbon[1], Nicole R. Saylor[1], Marete R. Seymour[1,2], Lance M. Shaner[1] and Patrick A. Gibney[2]

[1]Omega Yeast Labs, Chicago, IL, 60641; [2]Department of Food Science, Cornell University, Ithaca, NY,14853

Abbreviations: OYL (Omega Yeast Labs), UAS (upstream activation sequence), PCR (polymerase chain reaction), bp (base pairs)

Keywords: yeast, diastatic, diastaticus, *STA1*, refermentation, over-carbonation, dextrin

ABSTRACT

Diastatic yeasts are a major contamination risk to packaged beer. Brewery quality control programs rely on selective microbial growth media and molecular detection of the *STA1* gene, however there is a wide functional range of diastatic activity that remains unresolved by these current methods. Herein, we provide a comprehensive analysis of diastatic yeast selection media using our collection of *STA1+* strains. For the preparation of microbial selection media, we highlight several factors to control including plate age, autoclave cycling conditions, cupric sulfate and dipotassium phosphate concentrations. We further present several functional assays that confirm the variation in diastatic activity across *STA1+* strains and show a clear correlation with earlier onset and increasing strength in strains that contain an intact *STA1* promoter. Furthermore, we have developed robust and simple plate-based assay to confirm diastatic activity of potential contaminants. Collectively, these functional assays provide improved risk assessment of re-fermentation in packaged product with the potential for determining contaminant thresholds for different *STA1+* strains and weighing in on the decision to dump beer or issue a product recall.

INTRODUCTION

Diastatic strains of *Saccharomyces cerevisiae* are widely used industrial brewing strains, yet represent one of the major contamination threats to packaged beer. These strains are characterized by the ability to secrete a glucoamylase protein that degrades dextrins. Dextrins are mixtures of D-glucose polymers derived from grain starches in brewing. The dextrin composition of wort is influenced by the use of different malts and adjuncts and the mashing conditions; thus, dextrin levels can vary significantly between beer styles. The glucoamylase enzyme produced by diastatic strains breaks dextrins down into individual glucose molecules, which can then be consumed by diastatic and non-diastatic yeasts, leading to issues described below. Cross contamination of traditional ale or lager beers with a diastatic strain of *S. cerevisiae* poses risks of hyperattenuation and refermentation resulting in unwanted increased production of alcohol and CO_2. In packaged product, diastatic contamination can result in gushing beer and exploding bottles/cans. Diastatic strains of *S. cerevisiae* are the traditional yeast used in Belgian beer styles such as Saison, Belgian Golden Strong, and Biere de Garde. Among 1,011 *S. cerevisiae* isolates sequenced from wild, clinical and industrial sources, diastatic yeast were only found within a subset of industrial beer yeast and small group of isolates in French Guiana (Krogerus *et al.* 2019). Thus, these diastatic strains represent a rare sub-population of *S. cerevisiae* that are ubiquitous in the brewing industry, but are far less common among the overall *S. cerevisiae* population. These strains have mistakenly been called *S. diastaticus*, which incorrectly indicates that they are a non-*cerevisiae* species. These strains are often referred to as *S. cerevisiae var. diastaticus*, which inappropriately designates them as a single variant, and not a collective grouping of diastatic yeasts with a wide range of genotypic and phenotypic traits. This nomenclature issue is analogous to flocculent brewing strains, which also have the shared phenotype of flocculation among genetically and phenotypically diverse strains. For simplicity and clarity, we refer to these diastatic strains as *STA1+* strains of *S. cerevisiae*.

The genetic determinants for diastatic activity in *S. cerevisiae* are the *STA1, STA2,* and *STA3* genes. These genes are unlinked and reported positions are on Ch. V, II, and XIV, respectively (Pretorius *et al.* 1988). Absence of *STA1* correlates with loss of diastatic activity, and disruption of the *STA1* promoter decreases diastatic activity, both suggesting that *STA1* encodes the major, functional diastatic enzyme (Krogerus *et al.* 2019). However, another study has demonstrated that disruption of *STA2* also ablates diastatic activity, highlighting that this phenotype is complex and incompletely understood (Pretorius et al. 1986). The *STA1/2/3* genes arose from a gene fusion event between the *FLO11* and *SGA1 (*Yamashita *et al.* 1987*;* Lo and Dranginis 1996). *SGA1* is non-essential, intracellular α-1,4 glucoamylase expressed under sporulation conditions and is typically inactive in industrial brewing strains (Pugh and Clancy 1990). *FLO11* encodes a GPI-anchored cell surface glycoprotein that is induced in late stages of fermentation and plays a major role in flocculation. The *STA* genes contain the upstream regulatory sequence of *FLO11* along with the *FLO11* signal sequence for secretion fused to the α-1,4 glucoamylase of *SGA1* (Gagiano et al. 1999, Kim et al. 2004; Adam et al. 2004). The chimeric *STA1* gene encodes a secreted α-1,4 glucoamylase that is expressed during fermentation. This results in the degradation of dextrins in wort and hyperattenuated beers with low residual extract and elevated ethanol.

Within the *STA1+* industrial brewing strains, there is considerable variation in attenuation levels. A recent study identified and characterized a 1,162 bp deletion upstream of the *STA1* gene (Krogerus et al. 2019). This deletion removes key upstream activation sequences (UAS) and results in a decrease in *STA1* gene expression and also diastatic activity. For simplicity, we will further refer to strains containing an intact *STA1* gene as *STA1+*, and strains lacking the *STA1* gene as *sta1-*. We will refer to strains containing the 1,162 bp deletion as *uas*Δ, and strains without this deletion as *UAS+*. This *UAS* deletion is easily detected by PCR and can be used as a screening tool for brewer's when characterizing a house diastatic yeast or an unknown diastatic contaminant. Though PCR genotyping provides a valuable characterization and screening tool for brewers, there are limitations. First, there is considerable variability in diastatic activity among *STA1+* strains that is not explained solely by the 1,162 bp deletion in the *STA1* promoter. Second, PCR genotyping is susceptible to user error and extraneous variables that can lead to false-positive and false-negative results. Third, PCR results can only confirm the presence of DNA and does not determine whether the source of the DNA is a living cell. Lastly, there is the potential for a brewing contaminant to produce a dextrin-degrading enzyme unrelated to the *STA* genes. For example, a *Brettanomyces bruxellensis* strain was isolated from an overattenuated beer and found to produce both extracellular and intracellualar enzymes capable of degrading dextrins (Kumara *et al*. 1993).

This study set out to provide better tools for brewers to use in the detection and risk assessment of diastatic yeast contaminants. First, selection media for diastatic *S. cerevisiae* were evaluated by screening our *STA1+* collection on commercially available LCSM and FPDM. Based on observed variability, we developed an Omega-optimized LCSM recipe. We next developed two independent functional assays to test for diastatic activity in the brewery environment, and confirmed a range of diastatic activities. The onset and strength of diastatic activity both increased in *STA1+/UAS+* strains relative to *STA1+*/*uas*Δ strains. Finally, we also developed a simple plate-based assay that clearly distinguished *STA1+/UAS+* and *STA1+*/*uas*Δ strains. Each of these screening assays can be used to determine the diastatic activity of an unknown contaminant or as a complementary approach to verify PCR results. Lastly, we provide evidence that these assays correlate well with re-fermentation studies in packaged product and can be used to assess risk of a potential diastatic contaminant. Taken together, this work presents three new, validated methods to detect diastatic activity to aid the brewing industry with identifying and preventing contamination of their products.

EXPERIMENTAL METHODS

Yeast Strains

Industrial brewing strains used in this study are referenced by their commercial name supplied by Omega Yeast labs, LLC. A full summary of the strains can be found in Table 1.

PCR Genotyping of *STA1* and the 1,162 bp Deletion in the *STA1* promoter

Multiplex PCR was performed using the previously published primers SD-5A and SD-6B for *STA1* gene 868 bp fragment (Yamauchi *et al.* 1998) and STA1_UAS_Fw and STA1_UAS_Rv for the *STA1 UAS* 599 bp promoter fragment (Krogerus *et al.* 2019). Genomic DNA was prepared using an LiAc/SDS extraction (Looke *et al*. 2011). Briefly, roughly one colony was resuspended in 50 µL of 0.2M LiAC/1%SDS buffer solution and heated to 70°C for 5 minutes. The solution was precipitated with 200 µl of 100% EtOH at -20°C for 10 minutes. DNA was pelleted at 13,000 r.p.m. for 3 minutes, rinsed with 70% EtOH and air dried. The pellet was resuspended in 50 µl of DNAse/RNAse free water (Gibco). PCR reactions included 2 µl of DNA, 1 µM each primer, and 2x Gotaq mastermix (Promega). Touchdown PCR was performed (Korbie and Mattick 2008). Resulting PCR status was reported as *UAS*+ and *STA1*+ if the corresponding 599 bp and 868 bp bands were detected.

Preparation of Omega-optimized LCSM Selection Medium and Serial Dilution Plating for Comparing Commercial Formulations

Media formulations selected for comparison: Omega-optimized LCSM (0.4% yeast extract, 0.2% dried malt extract, 0.2% yeast peptone, 0.1% dipotassium ortho-phosphate, 0.05% ammonium sulfate, 0.06% cupric sulfate, 1% dextrose, 2% agar), commercial LCSM (Weber Scientific), and FPDM (Weber Scientific). YPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% agar) was used as a nutrient medium to compare growth and recovery of the serial diluted cultures. Plates were autoclaved together using a solid load setting for 20 minutes (Hiyarama HVA-110) and poured 24 hours, 48 hours or 96 hours prior to plating serial dilutions of the indicated strains. Serial dilutions of 1:6 were performed from a starting concentration of 1 million cells/ml with 5 µl of diluted cells spotted. Plates were imaged after 3 days at 30°C.

Bromocresol Green Maltodextrin (BGM) Medium Preparation

Liquid BGM (bromocresol green maltodextrin medium) is used to assess dextrin degradation based on using maltodextrin as a carbon source coupled with a pH indicator dye that changes color as yeast cells metabolize the maltodextrin. Components include maltodextrin at 2% (w/v; Sigma-Aldrich, #419672), yeast nitrogen base with ammonium sulfate at 0.67% (w/v, Sunrise Science Products, #1501-100), K_2HPO_4 at 0.014% (w/v; J.T. Baker, #4012-05), and bromocresol green at 0.022% (w/v; BBL, #04-198). The medium was then brought to a pH value of 5.8 - 6.0 using 1M HCl. Medium was then vacuum filter sterilized with a 0.2 µm PES filter. The ability of strains to degrade dextrin was tested using BGM. Isolated colonies grown on YPD agar were directly inoculated into 500 µl of BGM medium in a sterile 1.5 mL microcentrifuge tube. Tubes were capped and rotated at 70 r.p.m. at room temperature for up to 20 days. Color change was assessed in two different ways. First, a visual estimation was performed to determine whether the color was blue, green, or yellow. This estimation was used for table displays as shown in Figures 2 and 3. Second, color change was quantified by collecting cells by centrifugation, then removing 100 µL of the media to a 96-well plate and measuring absorbance at 620 nm in a UV-

Vis plate reader (Biotek Epoch2). Biological triplicates were performed using a separate colony for each replicate. To test the lower limit of detection in pure culture, 10-fold serial dilutions of three *STA1+* strains with varying diastatic activity (OYL-026, OYL-027 and OYL-019) were prepared to inoculate BGM with 100 - 1,000,000 cells. Similarly, the lower limit of detection was evaluated in mixed culture by mixing these *STA1+* strains in different ratios with a *sta1-* strain (OYL-006, British Ale I). In both cases, visual and quantitative assessment of color change was performed as described above.

Quantification of Dextrin Type III Degradation Using Iodine Staining

This assay is based on absorbance of iodine-stained starch. Individual colonies from YPD plates were inoculated into YPEG liquid medium (1% yeast extract, 2% peptone, 2% ethanol, 3% glycerol) + 2% Dextrin type III (MP Biomedicals) and incubated overnight at 23°C with agitation. Cultures were removed from the shaker and incubated without agitation at 23°C for 4 weeks. Dextrin degradation was measured at day 7, 14, 21 and 28. Each week, 200 µL of the culture was removed and briefly centrifuged to remove cells. 20 µL of the supernatant was transferred to a 1 cm cuvette with 1 mL of 0.04% Lugol's iodine solution (10% potassium iodide, 5% iodine). Measurements for absorbance were collected at 680 nm using a UV-vis spectrophotometer (Thermo Scientific GENESYS 180). The UV-vis spectrophotometer was blanked with 0.04% Lugol's iodine. A *sta1-* strain (OYL-088) was used as a negative control to normalize the *STA1+* strain measurements and determine the percentage of Dextrin type III remaining. The results are averaged from 8 biological replicates. Reported for each strain is the week at which Dextrin type III levels was reduced to <50% of starting Dextrin type III (as determined by the negative control OYL-088).

Maltodextrin Cross-feeding Assay to Detect Diastatic Activity

Maltodextrin plates were prepared using a synthetic complete minimal media formulation (Sunrise Science; 0.67%YNB+nitrogen, 0.2% SC-Complete mix, 2% agar) and 2% maltodextrin (Spectrum Chemical). Strains grown on YPD agar were patched to the maltodextrin plates adjacent to W303. The plates were incubated at 30°C aerobically for 1 month of observation. Strains were scored based on the following criteria; - (no difference to compared to OYL-088), + (growth of *STA1+* strain), ++ (growth of *STA1+* strain and supported growth of W303), and +++ (growth of *STA1+* strain with red pigment in W303). Limit of detection assays were performed with a spread plate of W303 and spotting of 20 µL test culture. The test cultures were from saturated propagations. The *STA1+/UAS* strain (OYL-026) was serially diluted 1:10 into the *sta1-* control strain (OYL-088). The corresponding concentration of cells is $2x10^6$ cells/spot *STA1+/UAS* strain (10^0) down to 2-3 cells/spot *STA1+/UAS* strain and $2x10^6$ cells *sta1-* strain (10^{-6}).

Bottle Refermentation and Analysis

Strains from YPD agar were inoculated into 2 ml YPEG liquid media and incubated overnight at 23°C with agitation. Saturated cultures were inoculated into 355 mL bottles of Lagunitas IPA at 3 million cells/bottle. Crowns were removed from bottles, the culture was inoculated and the bottles were recrowned. Negative controls included a *sta1-* strain (OYL-088) and a mock inoculation with water. A set of 10-fold dilutions was also performed with OYL-026, OYL-056, OYL-027 and OYL-042. The starting 3 million cells/bottle was serially diluted 1:10 in sterile water and the resulting dilutions were inoculated into bottles. Bottles were incubated at 30°C for

one month. The residual extract and alcohol by volume were measured using a DMA™ 4500 M and Alcolyzer ME (Anton Paar). Each inoculation was performed in biological triplicate.

RESULTS AND DISCUSSION

Evaluation and Optimization of Selection Media for *STA1+* Strain Detection

The most widely used *STA1+* strain selection media, Lin's Cupric Sulfate Medium (LCSM), is permissive for strains that have elevated copper resistance (Lin Y. 1981). This strongly correlates to the *STA1+* strains within the Beer II Group, as these are more closely related to industrial wine strains that have adapted resistance to copper-based fungicides (Galone *et al.* 2016). The recently introduced "Farber Pham Diastaticus Media" (FPDM) is suggested to be superior to LCSM for the detection of *STA1+* strains and additionally allows for differentiation of *STA1+* strains by starch degradation and a zone of clearance. The exact composition of this medium has not been reported.

We set out to optimize LCSM with the goal of having high recovery for *STA1+* strains and minimal growth of *sta1-* strains. We first prepared LCSM according to the original published formulation (Lin Y. 1981), and then performed a variety of tests to determine the ideal concentrations of cupric sulfate and dipotassium ortho-phosphate by comparing growth of strains in our catalog of *STA1+* and *sta1-* brewing strains (data not shown). Concentrations of 0.06% cupric sulfate and 0.01% dipotassium ortho-phosphate were ideal for selective growth of our *STA1+* strains (Figure 1). To denote the difference compared to the original recipe, we named our recipe Omega-optimized LCSM.

To assess the recovery rates and background growth of *sta1-* strains, serial dilutions were performed. All strains were plated on YPD agar to represent non-selective conditions controlling for equal plating and comparable growth rates (Figure 1A). *STA1+* and *UAS+* genotyping of the OYL strains was determined by PCR (Table 3). We grouped the strains in *STA1+/UAS+* (Group I), *STA1+/uasΔ* (Group II) and strains that have high levels of background when copper sulfate concentrations are reduced (Group III). Figure 1B shows the comparison of commercial LCSM, FPDM and the Omega-optimized LCSM. Commercial LCSM was the most stringent selection media with recovery of <50% of the Omega *STA1+* strains (OYL-026, OYL-033, OYL-112, OYL-500, OYL-501 and OYL-042) and no background growth of *sta1-* strains. Commercial LCSM also resulted in a lower limit of detection with 0.5-20% recovery rates in OYL-033, OYL-112, OYL-500, OYL-501. FPDM exhibited the greatest effect of plate aging with ~36-fold less cell growth from 24 to 48 hours, with no permissible growth after 96 hrs. Manufacturers advise using plates within 24 hours of pouring. FPDM also showed the highest background growth with 100% of the Group III *sta1-* strains exhibiting growth at the 1 million/ml plated cell density. With our Omega-optimized LCSM, we were able to recover all Omega *STA1+* strains. With the exception of OYL-040, we observed 100% recovery to the last serial dilution spot (~3 cells) if used within 48 hrs. The media minimizes background growth, but there is low-level, leaky growth of *sta1-* OYL-024 and OYL-030 Belgian strains. The Omega-optimized LCSM is effective after 96 hours, but shows slightly higher stringency.

With these results we conclude that while LCSM is a suitable selection media for *STA1+* containing strains, attention must be paid to the cupric sulfate and dipotassium ortho-phosphate concentrations, autoclave cycling, age of the plates and the potential for background observed in

a limited number of *sta1-* strains. Of the formulations tested, the Omega-optimized LCSM had the best performance. Of note, these *STA1+* selection media showed no difference in growth between *UAS+* and *uas*Δ strains (Figure 1). These results are not unexpected, as LCSM is based on the observation that copper resistance has been linked to presence of the *STA1* gene, but the mechanism of copper resistance is unrelated to the *STA1* gene or its product (Strope *et al.* 2015, Gallone *et al.* 2016). Thus, copper resistance doesn't necessarily predict diastatic activity, and screening with this medium could result in identification of a strain that lacks diastatic activity (*sta1-*, false positive) or displays significantly reduced diastatic activity, as is the case for some of the *STA1+*/*uas*Δ strains. We therefore explored further methods to characterize differences between these *STA1+* strains.

Methods for Functionally Assessing Diastatic Risk of STA1+ Strains

Few methods exist for the direct phenotypic evaluation of the diastatic activity of *STA1+* strains (Meier-Dörnberg *et al.* 2018, Krogerus *et al.* 2019). Diastatic activity varies widely among *STA1+* strains and recently has been shown to strongly correlate with a naturally occurring 1,162 bp deletion within the *STA1* promoter region (Krogerus *et al.* 2019). Brewers are faced with a decision to recall or destroy beer that has a confirmed *STA1+* PCR result or a contaminant that shows copper sulfate resistance, though because of this strain-to-strain variability the real risk of this result leading to refermentation and exploding bottles is unknown. Within our own observations and repeated accounts of false positive and negative rates from our brewery customers, we set out to develop robust, easy-to-use methods that breweries could use to routinely and accurately test yeast strains for diastatic (dextrin-degrading) activity. Our goal was to develop a number of different assays, providing flexibility so that brewers could use an assay that works best within their current production scheme and accurately assess spoilage risk. Ultimately, we developed three independent methods as described below.

Method #1: Monitoring pH as a Readout of Maltodextrin-Supported Growth

Instead of using correlation with copper resistance as a proxy for presence of the *STA1* gene, we sought to develop methods that directly assess diastatic activity. The first assay relies on pH change as an indicator of growth in medium with maltodextrin supplied as the only carbon source. Initial formulation trials suggested a liquid assay would perform better than a plate-based assay, as the plate-based assays had significant background growth even for strains lacking *STA1* (data not shown). This medium contains bromocresol green, which starts as dark blue, but changes to green and eventually yellow as growing yeast cells acidify the medium. A schematic illustrating how to perform this assay for detection of diastatic *S. cerevisiae* is shown in Figure 2A. A panel of OYL commercial brewing strains was tested using this assay and analyzed both visually and spectrophotometrically (Figure 2B, 2C). The *sta1-* strains, OYL-006 and OYL-088, both failed to acidify the medium as expected (Figure 2B, 2C). In contrast, we observed three general behaviors: strong diastatic activity (medium was turned completely yellow within 3 days), moderate diastatic activity (medium was turned completely yellow within 8 days), and weak diastatic activity (color change was incomplete) (Figure 2B, 2C). While most of the *STA1+*/*UAS+* strains had strong diastatic activity, OYL-033 exhibited moderate diastatic activity (Figure 2B, 2C). *STA1+/uasΔ* strains exhibited highly variable diastatic activity, and could be found with high, moderate, and weak activity (Figure 2C). Of note, the OYL-040 strain exhibited

the weakest diastatic activity, requiring incubation of >12 days to observe color change of the medium. These results highlight that there are likely genetic determinants beyond the presence of *STA1* and the *uasΔ* that determine diastatic activity.

Finally, we sought to determine how well this assay would perform in mixed culture to estimate our limit of detection for identifying a diastatic strain in a population of non-diastatic yeast (as might be found in a brewing situation). Again, results were assessed both visually and spectrophotometrically (Figure 3). Three strains were tested at different cell concentrations, both alone and in mixed culture with a *sta1-* strain (British Ale, OYL-006). The *STA1+/UAS+* French Saison (OYL-026) strain was able to acidify the medium even with 100 cells, though when mixed with a non-diastatic strain it was undetectable if diluted less than 1:10 (Figure 3). Two *STA1+/uasΔ* strains were also tested (Belgian Ale OYL-019 and Belgian Saison OYL-027). Both strains performed moderately when alone, though with slower kinetics than OYL-026 (Figure 3). As with OYL-026, they performed less well in mixed culture. These results suggest that this assay is not ideal for testing mixed culture, unless there is a very high ratio of diastatic cells. Rather, this assay is ideal for testing pure culture isolates to confirm whether or not they possess diastatic activity.

Method #2: Quantification of Dextrin Type III Degradation Using Iodine Staining

The second assay characterizes the potential risk of the different diastatic *S. cerevisiae* strains based on direct quantification of dextrin degradation. The Omega *STA1+* collection along with a *sta1-* control (OYL-088) were assayed for starch degradation over time. Colonies were cultured overnight with agitation in YPGE + 2% Dextrin III media to promote growth while relieving any glucose repression of *STA1* transcription (Kartasheva *et al.* 1996). The cultures were then left still on the benchtop for up to one month to monitor Dextrin III degradation. Iodine was used to detect the remaining Dextrin type III in each sample using absorbance at 680 nm. A schematic illustrating this assay is shown in Figure 4A. Summarized in Figure 4B is the timing of starch breakdown in the various *STA+* strains. Several highly diastatic strains such as OYL-026, OYL-056, OYL-205, OYL-500 and OYL-501 were able to degrade >50% of the Dextrin III by week 2. These strains also contain an intact *STA1* promoter region (*UAS+*), and thus are predicted to have higher *STA1* expression and diastatic activity. This wasn't always the case with *STA1+*/*UAS+* strains, as OYL-033 and OYL-039 typically took up to 3 weeks to degrade >50% of the Dextrin III. Nonetheless, when assessing *STA1+*/*UAS+* strains relative to *STA1+/uas*Δ strains after 4 weeks, the *STA1+*/*UAS+* strains exhibited significantly more Dextrin type III degrading activity (Figure 4D). Variation in diastatic activity was observed in *STA1+/uas*Δ strains, with OYL-042 showing starch breakdown by 3 weeks and other strains such as OYL-019, OYL-200 and OYL-040 rarely reaching >50% dextrin breakdown by week 4. In fact, when the assay was terminated the average amount of remaining starch for OYL-040 was 93% (Figure 4C). OYL-019, OYL-040 and OYL-200 measurements were carried out to 8 weeks to investigate this further. Eventually OYL-019 and OYL-200 reached 50-60% dextrin breakdown, where 80-100% of the original dextrin levels remained in OYL-040 (data not shown). The *STA1* gene from OYL-040 was sequenced to identify potential genetic changes that could explain how a *STA+/UAS+* strain exhibited such low diastatic activity. Multiple SNPs were observed in OYL-040 *STA1* that were distinct from reported *STA1* sequences of *STA1+*/UAS+ and *STA1+/uas*Δ (Krogerus *et al.* 2019), including two amino acid changes

(S310L and V668P) (Figure 4E). Further experimentation is needed to determine whether these unique OYL-040 SNPs could lead to an altered Sta1 protein and lessened diastatic activity. This highlights the potential for other strain-specific genetic alterations that further distinguish *STA1+* strains and their respective diastatic risk.

Method #3: Maltodextrin Cross-feeding Assay to Detect Diastatic Activity

A major risk that diastatic strains pose to packaged beer is the potential for a small amount of diastatic cells to secrete the glucoamylase enzyme, which then degrades starch over time and provides simple sugars for yeast to ferment. This premise was used to design a cross-feeding assay to score diastatic activity. *STA1+* strains were patched adjacent to a *sta1-* indicator strain on plates containing maltodextrin as the sole carbon source and scored for *STA1+* supported growth of the *sta1-* indicator strain. We predicted that if the *STA1+* strain secreted high levels of Sta1 protein, then the enzymatic breakdown of maltodextrin would support the growth of a *sta1-* indicator strain (Figure 5A). The *sta1-* indicator strain used was the lab strain, W303, containing the *ade2-1* allele, which results in the accumulation of the intermediate metabolite P-ribosylaminoimidazole carboxylate (red pigment) into vacuoles and the development of red colonies. This strain choice helped differentiate between robust growth (red), intermediate growth (red-white gradient) and slow growth (white) of the *sta1-* strain (Figure 5B, 5C). The Omega *STA1+* strains were assayed with the criteria illustrated in Figure 5C and the results are summarized in Table 2. In just one week of growth, the *STA1+/UAS+* strains were able to support intermediate to robust growth of the W303 strain. Only a subset of *STA1+/uas-* supported intermediate growth, others exhibited slow growth without the cross-feeding of the W303 strain, and the OYL-040 strain was indistinguishable from the *sta1-* control, OYL-088 (Figure 5B). Growth was scored again after 30 days and still several of the *STA1+/uas-* strains (OYL-019, OYL-025 and OYL-040) were unable to provide enough usable glucose to cross-feed and promote color change in the W303 *sta1-* indicator strain (Table 2). These results correlate very well with Methods 1 and 2 and again we observed OYL-040 was the least diastatic relative to the *STA1+/uas*Δ strains. Within a week of growth, this assay strongly differentiates between *STA1+/UAS+ and STA1+/uas*Δ strains. Therefore, this assay can be used similar to PCR as a follow up for any suspected contaminant to further differentiate between strains that contain intact promoters and the 1,162 bp deletion. In addition, the varying diastatic phenotypes within *STA1+/UAS+ and STA1+/uas*Δ genotypes can be used to help assess risk of hyperattenuation.

The limit of detection for this assay was assessed as a method for screening yeast slurries, propagations or enriched beer samples. The culture/slurry was spotted onto a lawn of W303 cells and color change was scored at the perimeter of the spot. Unfortunately, not all strains exhibited robust enough diastatic activity to resolve contaminants below 1 million cells/ml. An enrichment approach in liquid LCSM media was employed to test this further. Overnight saturated YPGE cultures were diluted into liquid LCSM for a 24-hour enrichment. OYL-026 (*STA1+/UAS+)* was serially diluted 1:10 into OYL-088 (*sta1-)* inoculated cultures. The lowest dilution volume 10^{-6} corresponds to 200/ml cells of OYL-026 and $200x10^{6}$ cells/ml of OYL-088. 10 ul of the LCSM enrichments were spotted onto the YNB-SC maltodextrin plates with a lawn of the indicator strain W303. A color change in W303 indicating the presence of OYL-026 was observed down to the 10^{-6} dilution (Figure 5D). The turnaround of this assay was 7-9 days and may not be suitable for screening brewery propagations as this yeast would already be in production.

Bottle Refermentations Assess Diastatic Activity in Packaged Product

The phenotypic assays described here are in strong agreement with previous observations that there are high risk and low risk *STA1+* strains. To test this in packaged beer, *STA1+* strains were inoculated into a Lagunitas IPA bottles to mimic contamination at packaging. Lagunitas IPA was chosen as a representative ale, with a 2.6°P residual extract, moderate hopping rate and 6.2% abv. One-month post-inoculation, OYL-026, OYL-112 and OYL-056 strains lowered the residual extract to 1.3-1.6°P (Figure 6A). This represents an addition of >2 volumes of CO_2 in the packaged product, which at room temperature is above the pressure rating for a glass beer bottle and a significant hazard. The remaining *STA1+/UAS+* strains, excluding OYL-501, were >0.6°P below the OYL-088 *sta1-* control. In agreement with the other assays presented in this paper, OYL-501 displays lower diastatic activity relative to the other *STA1+/UAS+* strains. Collectively these results suggest OYL-501 may pose less risk to packaged product than other *STA1+/UAS+* strains. Interestingly, the residual extracts in OYL-019, OYL-025, OYL-031 and OYL-042 were also lowered by 0.4-0.6P (Figure 6A) despite the 1,162 bp promoter deletion. At this high rate of inoculant (10,000 cells/ml) these *STA1+/uas*Δ strains still pose a re-fermentation risk. The mean Plato and ABV of *STA1+/UAS+* and *STA1+/uasΔ* were compared to the *sta1-* control OYL-088 and mock water inoculation (Figure 6B and 6C). The *STA1+/UAS+* strains were significantly lower in Plato than the negative controls, whereas the *STA1+/uas*Δ strains were not. Several strains resulted in increased ABVs (0.4-0.6%) that are above the TTB tolerance for reported ABV (0.3%). These bottle experiments confirm that overall *STA1+/UAS+* strains are more hyperattenuative, however *STA1+/uas*Δ strains still pose a risk to packaged product.

To determine the minimum dose (CFU/ml) that results in re-fermentation of the packaged beer within a month, bottles were inoculated with 10-fold dilutions of the most potent *STA1+*/UAS+ (OYL-026 and OYL-056) and *STA1+/uas*Δ (OYL-027, OYL-042) strains (Figure 6D). After 1 month, the minimum dose of 10 cells/ml of OYL-026 and OYL-056 was sufficient to re-ferment 0.6°P and 0.2°P, respectively, whereas 10 cells/ml of OYL-027 and OYL-042 were not sufficient for re-fermentation. We predict that over extended periods of time, lower doses would result in re-fermentation. Additionally, we observed a dose response for increasing re-fermentation with increased number of cells, suggesting that thresholds for CFU/ml contamination levels each diastatic strain can be determined. It is important to note that the composition of the beer will have an impact on this as well. Factors such as the presence of additional viable *sta1-* yeast and the amount of dextrins/residual extract at packaging must be considered.

CONCLUSIONS

Herein, we provide a comprehensive analysis of selection media for diastatic strains of *S. cerevisiae* using our OYL collection of *STA1+* strains. We highlight several factors to control for including plate age, autoclave cycling conditions, cupric sulfate and dipotassium phosphate concentrations. Secondly, we confirm the variation in diastatic activity across *STA1+* strains and show a clear correlation with earlier onset and increasing strength in strains that contain an intact promoter (*STA1+/UAS+*). We also provide multiple robust and simple liquid- or plate-based

assays for the functional risk assessment of diastatic *S. cerevisiae* contamination. Overall, we propose that quality control for diastatic *S. cerevisiae* contaminations in breweries should include detection by a combination of verifying *STA1/UAS* status with PCR and/or functional assays described here. This information can be used to assess the risk of re-fermentation in packaged product with the potential for determining contaminant thresholds for different *STA1+* strains and weighing in on the decision to dump beer or issue a product recall.

Overall, the functional assays presented throughout this paper corelate medium-high diastatic activity with the presence of an intact promoter (*STA1+/UAS+*) as described previously (Krogerus *et al* 2019). In fact, the OYL collection of *STA1+/UAS+* scored medium-high diastatic activity across the board (Table 3). Our customers commonly report hyperattenuation and continued fermentation in package product with OYL-026, OYL-056 and OYL-500, which were among the highest scoring strains in these assays. One notable exception within the *STA1+/UAS+* strains, was OYL-501 which scored medium-high in our functional assays, but in the bottle refermentation experiment lacked diastatic activity. This result was surprising to our team and we repeated this several times to verify. However, consistent with reports from our customers, once OYL-501 finishes fermentation it is much less likely to result in re-fermentation in packaged product than its parent strain OYL-026. OYL-501 is a segregant from a genetic hybrid of OYL-026 (*STA1+/UAS+*) and the OYL-005 Irish ale yeast (*sta1-*). We predict that as a result, this strain is missing an unknown/undescribed genetic determinant important for maintaining diastatic activity in finished beer. Possibilities include surviving in finished beer, continuing to express *STA1*, or reactivating fermentation.

Within the group of *STA1+/uasΔ* strains, there was considerable variability from mild-medium diastatic activity in our functional assays. Without an intact promoter, the expression of *STA1* may be more variable/stochastic and result in both low and medium-risk diastatic strains. It is also possible that other unknown genetic determinants are at play and the low level of *STA1* expression is consistent within these strains, but the ability to survive and thrive allows for the range of activities described. An outlier within the *STA1+/uasΔ* strains was OYL-040, which had the lowest scoring diastatic activity in each of our assays. With this observation, we hypothesized that there was an additional inactivating mutation within the *STA1*-coding sequence in addition to the promoter deletion. We did not observe any deleterious mutations such as an insertion/deletion, frameshift or premature stop codon, however we did identify two single nucleotide polymorphisms (SNPs) that result in amino acid substitutions. Importantly, these OYL-040 *STA1* SNPs are novel and have not been reported in the *STA1* sequences that are publicly available (Krogerus *et al.* 2019). It is possible that these amino acid substitutions alter the activity of Sta1, however additional experimentation is necessary to confirm these SNPs result in the weakened diastatic activity observed in OYL-040.

In summary, these functional assays are cheap and accessible for brewery QC labs and provide two added benefits. First, they can easily be used to rule out false positive/negative results from traditional PCR/microbiological methods. Second, these functional assays can be used to assess the risk of potential contamination and factor in on the decision to destroy or recall product.

REFERENCES

Adam, A. C., Latorre-García, L. and Polaina, J. (2004) 'Structural analysis of glucoamylase encoded by the STA1 gene of Saccharomyces cerevisiae (var. diastaticus)', *Yeast,* 21(5), pp. 379-88.

Gagiano, M., Van Dyk, D., Bauer, F. F., Lambrechts, M. G. and Pretorius, I. S. (1999a) 'Divergent regulation of the evolutionarily closely related promoters of the Saccharomyces cerevisiae STA2 and MUC1 genes', *J Bacteriol,* 181(20), pp. 6497-508.

Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., Merlevede, A., Roncoroni, M., Voordeckers, K., Miraglia, L., Teiling, C., Steffy, B., Taylor, M., Schwartz, A., Richardson, T., White, C., Baele, G., Maere, S. and Verstrepen, K. J. (2016) 'Domestication and Divergence of Saccharomyces cerevisiae Beer Yeasts', *Cell,* 166(6), pp. 1397-1410.e16.

Kartasheva, N. N., Kuchin, S. V. and Benevolensky, S. V. (1996) 'Genetic aspects of carbon catabolite repression of the STA2 glucoamylase gene in Saccharomyces cerevisiae', *Yeast,* 12(13), pp. 1297-300.

Kim, T. S., Kim, H. Y., Yoon, J. H. and Kang, H. S. (2004) 'Recruitment of the Swi/Snf complex by Ste12-Tec1 promotes Flo8-Mss11-mediated activation of STA1 expression', *Mol Cell Biol,* 24(21), pp. 9542-56.

Kim, T. S., Lee, S. B. and Kang, H. S. (2004) 'Glucose repression of STA1 expression is mediated by the Nrg1 and Sfl1 repressors and the Srb8-11 complex', *Mol Cell Biol,* 24(17), pp. 7695-706.

Korbie, D. J. and Mattick, J. S. (2008) 'Touchdown PCR for increased specificity and sensitivity in PCR amplification', *Nat Protoc,* 3(9), pp. 1452-6.

Krogerus, K., Magalhães, F., Kuivanen, J. and Gibson, B. (2019) 'A deletion in the STA1 promoter determines maltotriose and starch utilization in STA1+ Saccharomyces cerevisiae strains', *Appl Microbiol Biotechnol,* 103(18), pp. 7597-7615.

Kumara, H.M.C.S., De Cort, S., Verachtert, H. (1993) 'Localization and characterization of alpha-glucosidase activity in *Brettanomyces lambicus'*, *Applied and Environmental Microbiology*, 59(8), pp. 2352-2358.

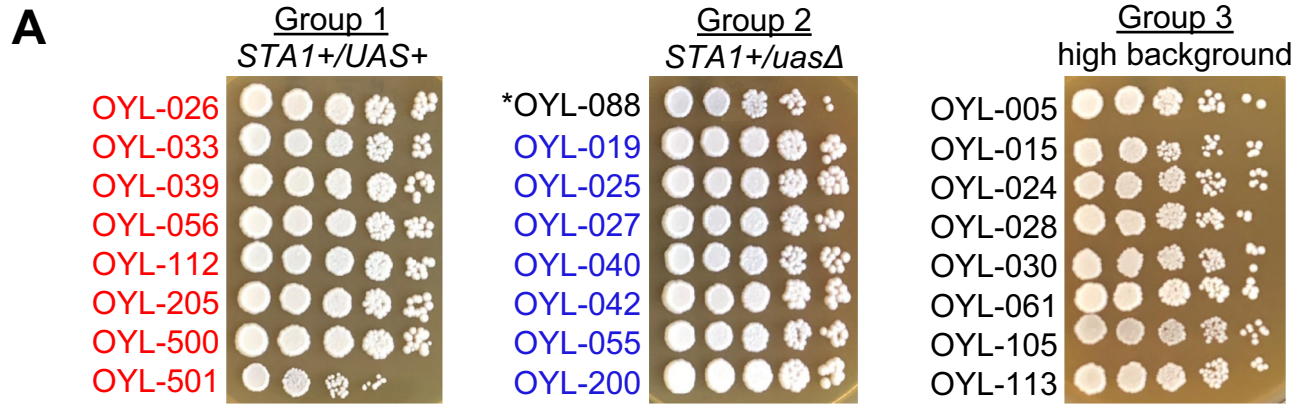
Lo, W. S. and Dranginis, A. M. (1996) 'FLO11, a yeast gene related to the STA genes, encodes a novel cell surface flocculin', *J Bacteriol,* 178(24), pp. 7144-51.

Meier-Dörnberg, T., Kory, O. I., Jacob, F., Michel, M. and Hutzler, M. (2018) 'Saccharomyces cerevisiae variety diastaticus friend or foe?-spoilage potential and brewing ability of different Saccharomyces cerevisiae variety diastaticus yeast isolates by genetic, phenotypic and physiological characterization', *FEMS Yeast Res,* 18(4).

Pretorius, I. S. and Marmur, J. (1988) 'Localization of yeast glucoamylase genes by PFGE and OFAGE', *Curr Genet*, 14(1), pp. 9-13.

Yamauchi, H., Yamamoto, H., Shibano, Y., Amaya, N., Saeki, T. 1998. 'Rapid methods for detecting Saccharomyces diastaticus, a beer spoilage yeast, using the polymerase chain reaction.' *Journal of the American Society of Brewing Chemists*. 56(2), pp. 58-63.

Figure Set (6 figures)



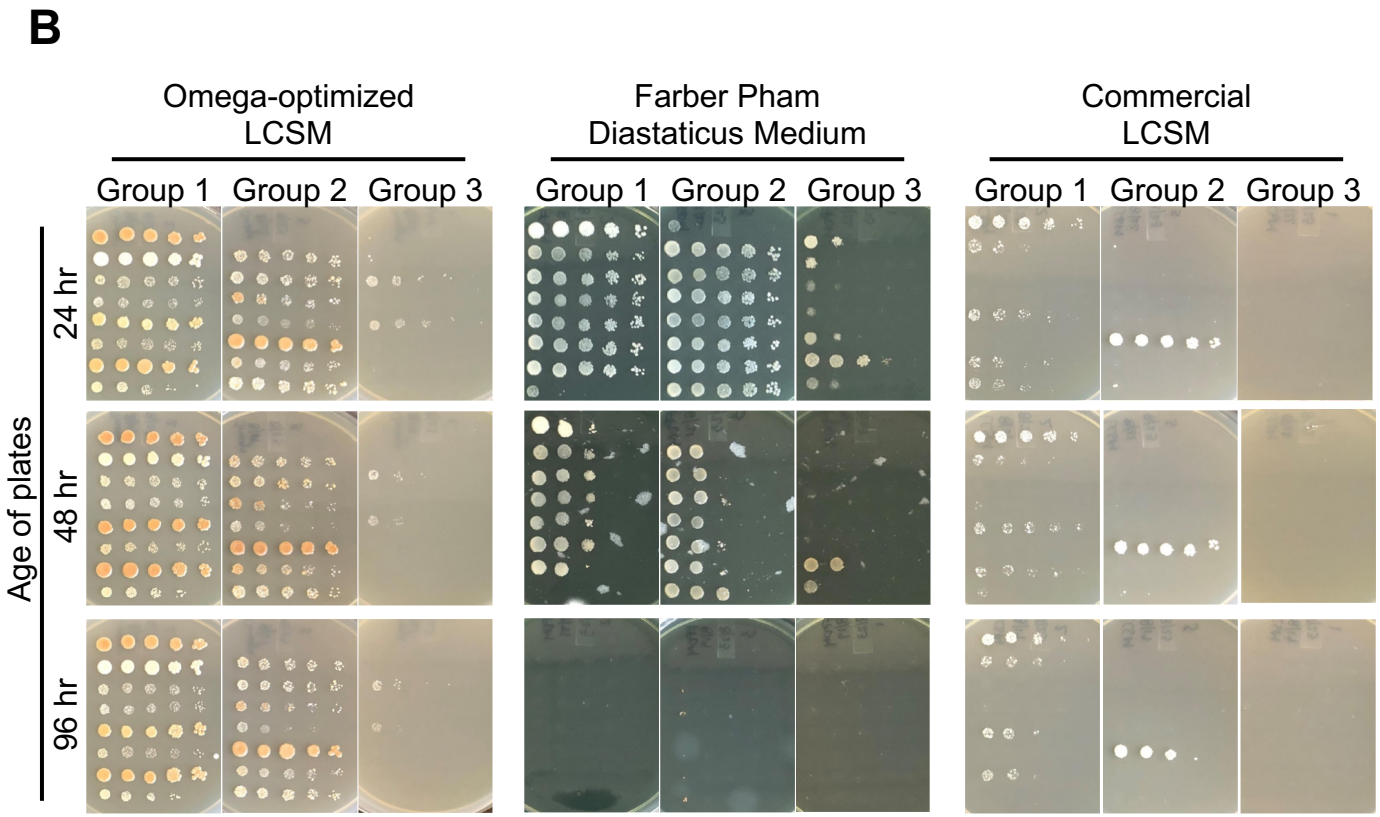


Figure 1. Comparison of diastatic selection media. **A**. Serial dilutions of indicated OYL strains were performed on nutrient rich medium (YPD). Group 1 strains are *STA1+/UAS+,* group 2 strains are *STA1+/uasΔ*, group 3 strains exhibit high background on LCSM. Serial dilutions of 1:6 were performed from a starting concentration of $1x10^6$ cells/ml. Plates were imaged after 3 days at 30°C. *OYL-088 is included as a control strain that does not contain the *STA1* gene. Strain numbers are colored as follows: red (*STA1+/UAS+*), blue (*STA1+/uasΔ*), and black (*sta1-*). **B**. Serial dilutions were performed as in panel A using indicated formulations of LCSM diastatic selection medium. Plates were autoclaved and poured 24, 48, or 96 hours prior to plating serial dilutions.

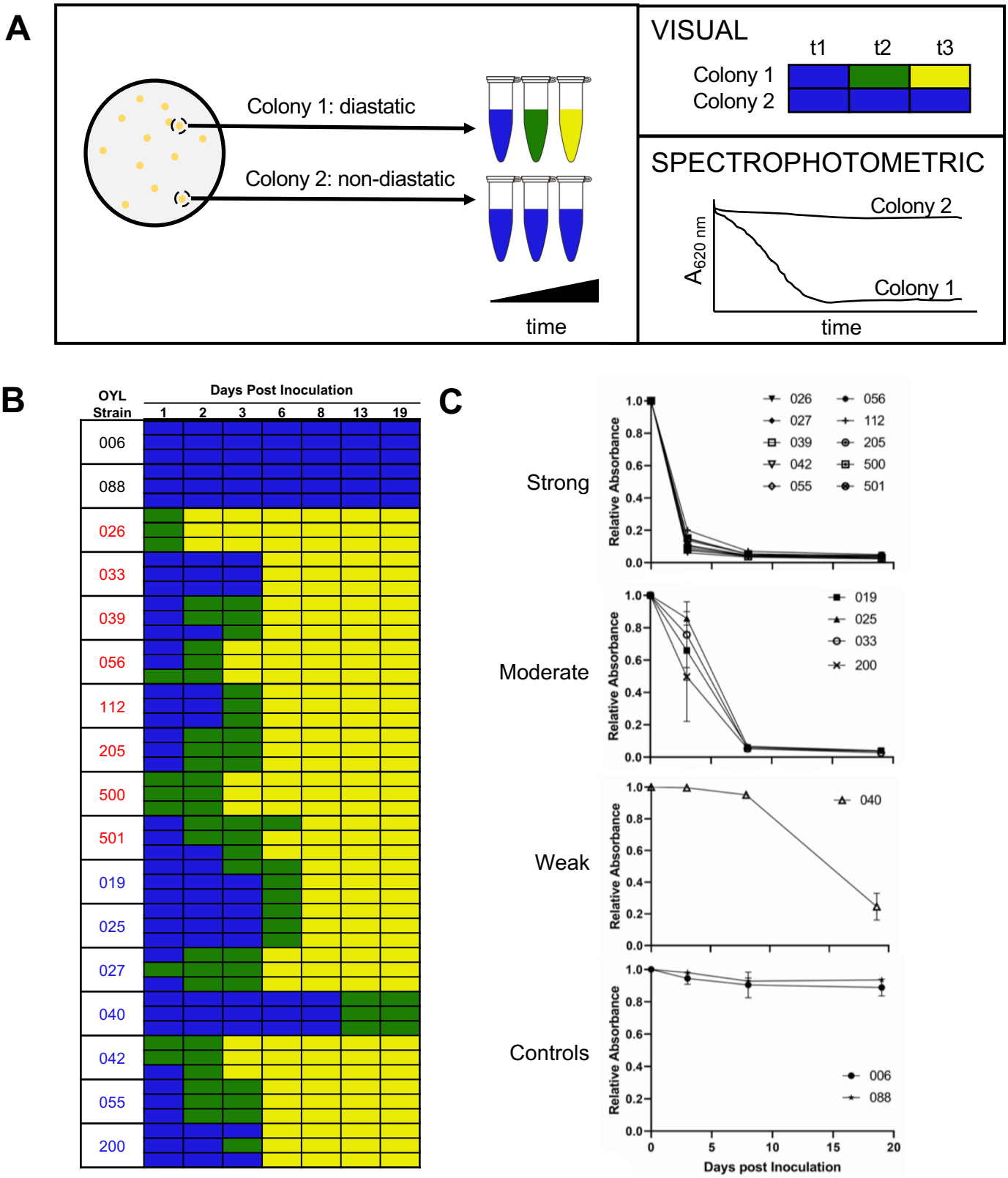
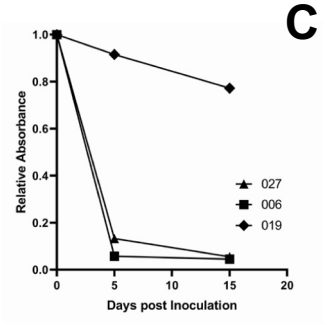


Figure 2. Monitoring pH as a readout of maltodextrin-supported growth using BGM medium. A. Illustration of two colonies picked from a plate with differing diastatic activity, both inoculated into BGM medium (blue) and subsequent color change over time for the colony with diastatic activity. **B**. Color change of the indicated strains grown in DDM assessed visually over time. Biological triplicates were performed for each strain. **C**. The relative absorbance at 620 nm of indicated strains grown in BGM medium. Strains are grouped based on the kinetics of media color change (complete conversion in 3 days, complete conversion in less than 8 days, partial conversion in 19 days, and strains that do not affect the medium color. Strain numbers are colored as follows: red (*STA1+*/*UAS+*), blue (*STA1+*/*uasΔ*), and black (*sta1-*).

A

Strain(s)	Cell numbers	Days post Inoculation 1	2	3	4	5	10	15
OYL-019	1,000,000							
	100,000							
	10,000							
	1,000							
	100							
OYL-019:OYL-006	1,000,000:1,000,000 *(1:1)*							
	100,000:1,000,000 *(1:10)*							
	10,000:1,000,000 *(1:100)*							
	1,000:1,000,000 *(1:1,000)*							
	100:1,000,000 *(1:10,000)*							
OYL-026	1,000,000							
	100,000							
	10,000							
	1,000							
	100							
OYL-026:OYL-006	1,000,000:1,000,000 *(1:1)*							
	100,000:1,000,000 *(1:10)*							
	10,000:1,000,000 *(1:100)*							
	1,000:1,000,000 *(1:1,000)*							
	100:1,000,000 *(1:10,000)*							
027	1,000,000							
	100,000							
	10,000							
	1,000							
	100							
OYL-027:OYL-006	1,000,000:1,000,000 *(1:1)*							
	100,000:1,000,000 *(1:10)*							
	10,000:1,000,000 *(1:100)*							
	1,000:1,000,000 *(1:1,000)*							
	100:1,000,000 *(1:10,000)*							

B



C

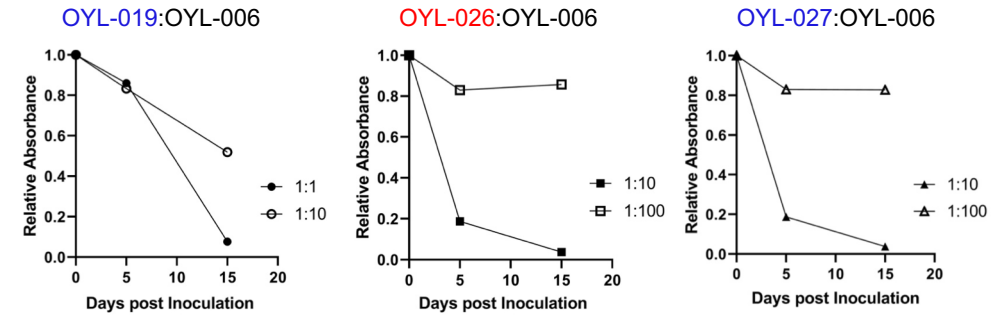
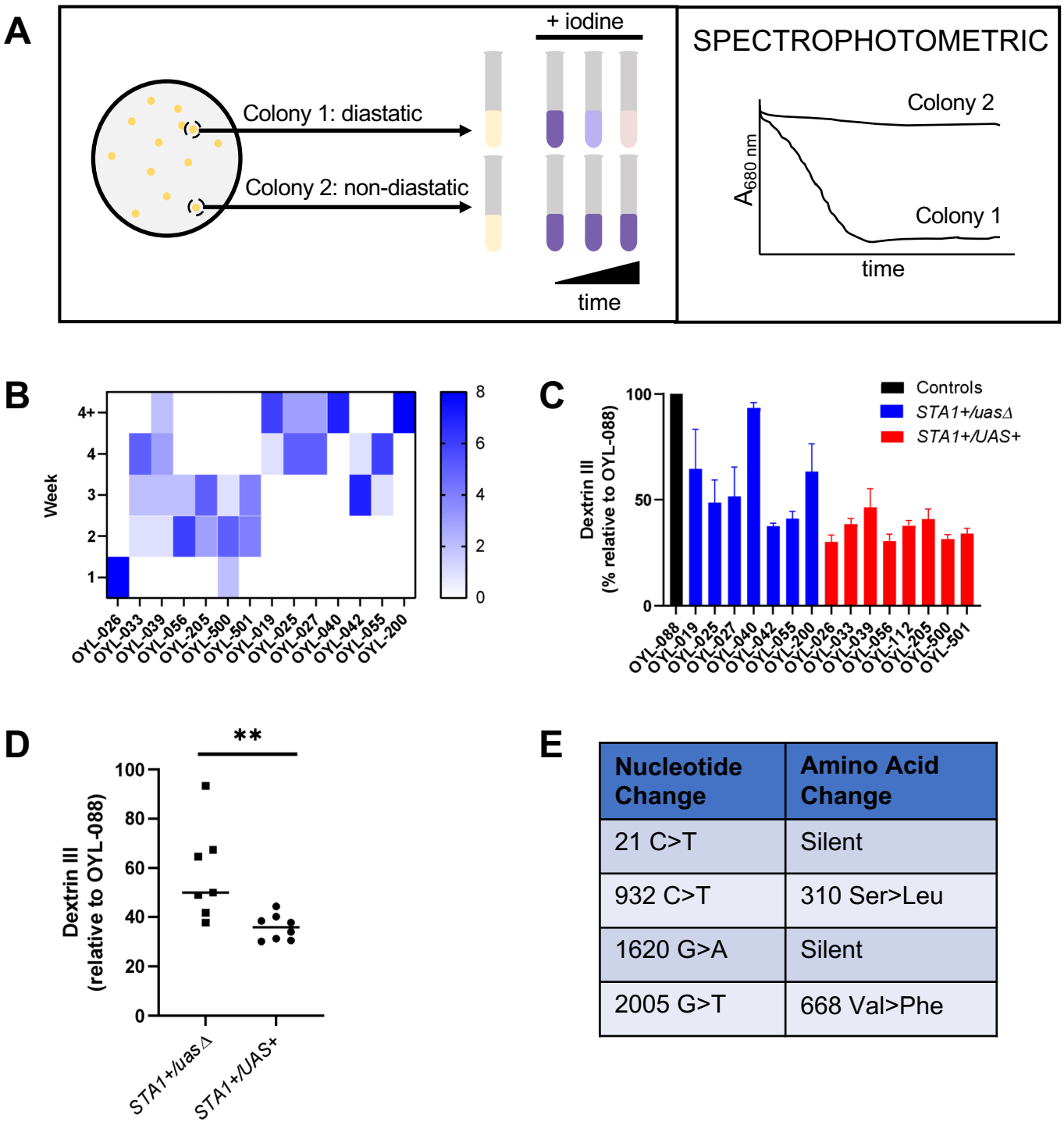


Figure 3. Assessing the lower limits of detection for BGM medium in pure and mixed culture. **A.** Visual assessment of detection limit for pure and mixed cultures for indicated strains with indicated number of cells. The lowest number of cells tested was 100, as indicated. **B**. Spectrophotometric assessment of BGM color change for lowest number of detectable cells in pure culture (OYL-019: 1,000 cells; OYL-026: 100 cells; OYL-027: 100 cells). **C**. Spectrophotometric assessment of BGM color change for mixed cultures (shown for each strain is the least detectable ratio to turn yellow within 15 days, and a 10-fold lower ratio). Strain numbers are colored as follows: red (*STA1+*/*UAS+*), blue (*STA1+*/*uasΔ*), and black (*sta1-*).



E

Nucleotide Change	Amino Acid Change
21 C>T	Silent
932 C>T	310 Ser>Leu
1620 G>A	Silent
2005 G>T	668 Val>Phe

Figure 4. Quantification of dextrin type III degradation using iodine staining . A. Schematic of dextrin quantification using iodine staining. **B.** Heat map representation for number of weeks required to reach >50% dextrin (type III) degradation. The scale bar indicates the number of experimental replicates. **C**. Dextrin (type III) remaining compared to *sta1-* control, OYL-088. Dextrin levels were measured after one month. Strains are categorized either as *UAS+* or *uasΔ*, as indicated. **D.** Quantification and statistical analysis of data shown in panel B. ** indicates $p<0.005$ by Student's t-test. **E**. Unique SNPs detected in OYL-040 by Sanger sequencing the *STA1* gene. Strain numbers are colored as follows: red (*STA1+*/*UAS+*), blue (*STA1+*/*uasΔ*), and black (*sta1-*).

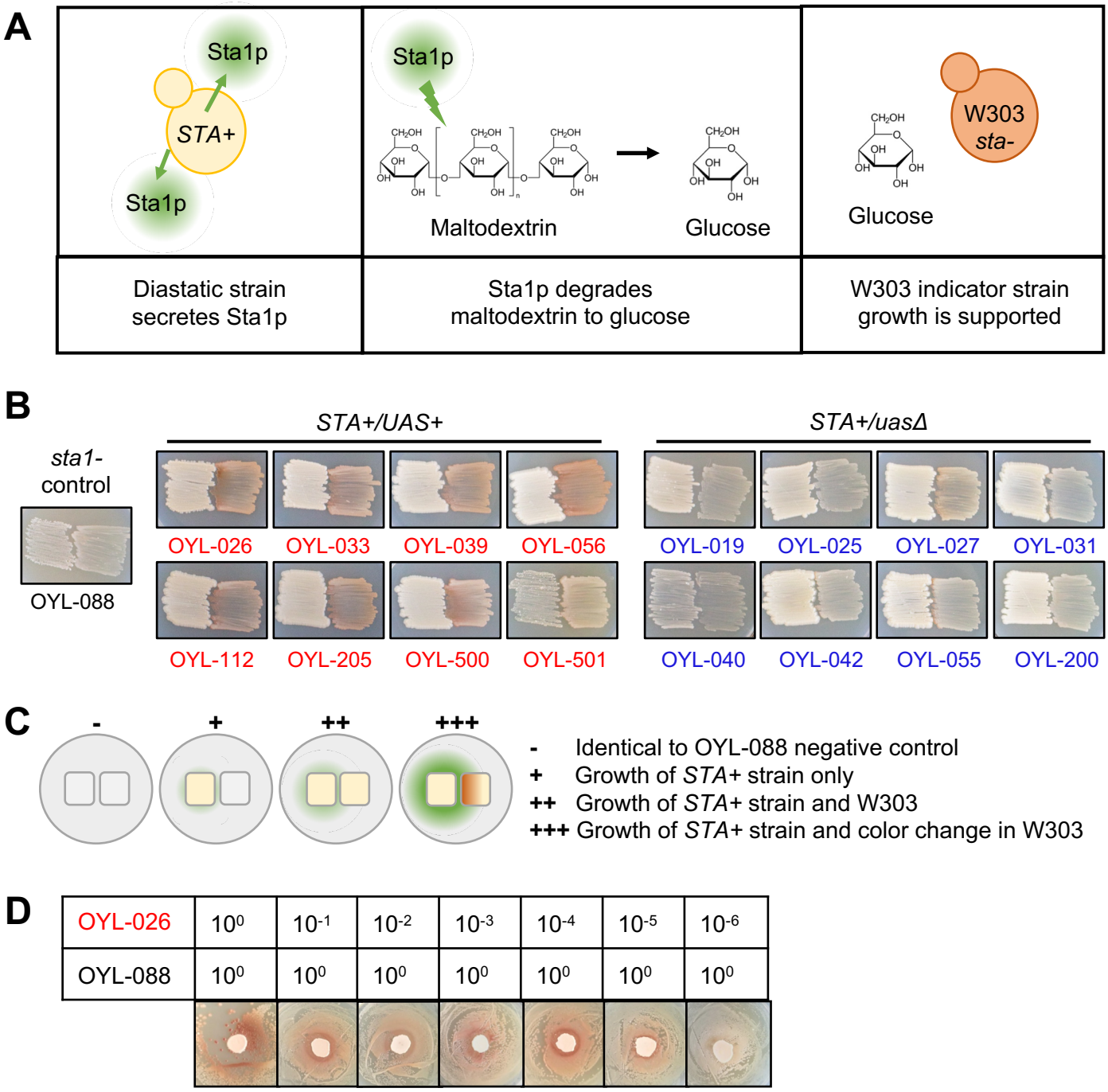


Figure 5. Maltodextrin cross-feeding assay for diastatic activity detection. **A**. Schematic of maltodextrin cross-feeding assay. Briefly, *STA+* cells secrete Sta1p, which degrades maltodextrin to glucose. Available glucose can be used by the W303 indicator strain, which turns red due to a mutation in the *ade2* gene. **B**. On each plate a *STA+* strain is patched on the left, and the W303 indicator strain is patched adjacent to the *STA+* strain on the right. Plates were incubated at 30°C for 10 days before photographing. **C.** Schematic of scoring system used to quantify maltodextrin co-culture assay. **D**. Limit of detection using maltodextrin co-culture assay was performed by dilution OYL-026 (*STA1+*/UAS+) as indicated into an OYL-088 (*sta1-*) culture, enriched in liquid LCSM and plated for a corresponding 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} of OYL-026 cells for every OYL-088. Red growth surrounding the colony shows W303 supported growth by OYL-026 diastatic activity. Strain numbers are colored as follows: red (*STA1+*/*UAS+*), blue (*STA1+*/*uasΔ*), and black (*sta1-*).

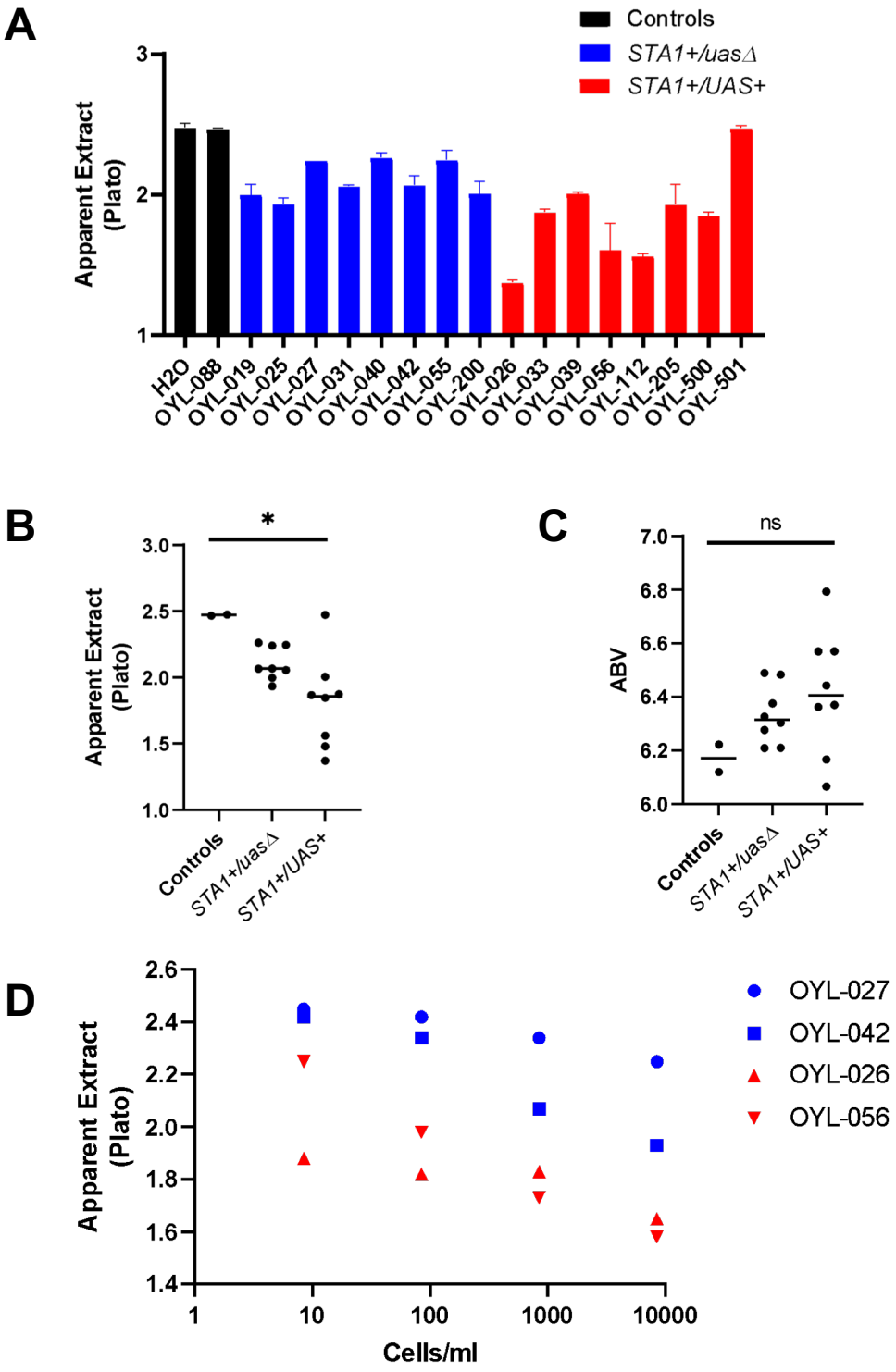


Figure 6. Beer refermentation with *STA1+* strains. **A**. Plato measurements after 1 month in Lagunitas IPA inoculated with 3 million cells/bottle of the indicated strain (or equivalent amount of water as a negative control). **B**. Plato measurement from panel A after grouping samples as controls (water only), *UAS+* strains, and *uasΔ* strains. (*indicates $p<0.05$ by one-way ANOVA) **C.** Alcohol by volume (ABV) for samples in panel A, grouped as shown in panel B. **D**. Plato measurements after 1 month in Lagunitas IPA inoculated with varying concentrations of the indicated strains. OYL-027 and OYL042 are *STA1+/uasΔ*, while OYL-026 and OYL-056 are *STA1+/UAS+*. Strain numbers are colored as follows: red (*STA1+/UAS+*), blue (*STA1+/uasΔ*), and black (*sta1-*).

3 tables

Table 1. Yeast strains used in this study

Strain	Description
OYR-001	W303-derived MAT**a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*
OYL-005	Irish Ale
OYL-006	British Ale I
OYL-015	Scottish Ale
OYL-019	Belgian Ale D
OYL-024	Belgian Ale A
OYL-025	Bavarian Wheat
OYL-026	French Saison
OYL-027	Belgian Saison I
OYL-028	Belgian Ale W
OYL-030	Wit
OYL-033	Jovaru
OYL-039	Biere de Garde
OYL-040	Belgian Dark Ale
OYL-042	Belgian Saison II
OYL-055	Vermont Farmhouse
OYL-056	Belgian Golden Strong
OYL-061	Voss Kveik
OYL-088	Neutral Ale
OYL-105	West Coast Lager
OYL-112	Swiss Ale
OYL-113	Mexican Lager
OYL-200	Tropical IPA
OYL-205	Specter Ale
OYL-500	Saisonstein's Monster
OYL-501	Gulo Ale

Table 2. Scoring of *STA1+* Strains in Maltodextrin Co-culture Assay

Strain	*UAS*	Day 3	Day 7	Day 8	Day 10	Day 30
OYL-088	-	-	-	-	-	-
OYL-019	-	-	+	+	+	+
OYL-025	-	-	+	+	+	+
OYL-027	-	+	+++	+++	+++	+++
OYL-040	-	-	-	-	-	-
OYL-042	-	+	+	+++	+++	+++
OYL-055	-	+	+++	+++	+++	+++
OYL-200	-	-	+	+	+	+++
OYL-026	+	+++	+++	+++	+++	+++
OYL-033	+	+	+++	+++	+++	+++
OYL-039	+	+	+++	+++	+++	+++
OYL-056	+	+	+++	+++	+++	+++
OYL-112	+	+	+++	+++	+++	+++
OYL-205	+	+	+++	+++	+++	+++
OYL-500	+	+++	+++	+++	+++	+++
OYL-501	+	-	++	++	+++	+++

- \- No difference to OYL-088
- \+ Growth of *STA+* strain
- ++ Growth of *STA+* strain and W303
- +++ Growth of *STA+* strain and color change in W303

Table 3. Comparison of *STA1*+ strains using different assays for diastatic activity

Strain	Description	STA1 PCR[A]	UAS PCR[B]	LCSM[C]	METHOD #1[C]	METHOD #2[C]	METHOD #3[C]	Bottle Referm[C]
OYL-006	British Ale I	-	-	nd	-	nd	nd	nd
OYL-088	Neutral Ale	-	-	No Growth	-	-	-	-
OYL-026	French Saison	+	+	Growth	+++	+++	+++	+++
OYL-033	Jovaru	+	+	Growth	++	++	++	++
OYL-039	Biere de Garde	+	+	Growth	+++	++	++	++
OYL-056	Belgian Golden Strong	+	+	Growth	+++	+++	++	+++
OYL-112	Swiss Ale	+	+	Growth	+++	+++	++	+++
OYL-205	Specter Ale	+	+	Growth	+++	+++	++	++
OYL-500	Saisonstein's Monster	+	+	Growth	+++	+++	+++	++
OYL-501	Gulo Ale	+	+	Growth	+++	+++	++	-
OYL-019	Belgian Ale D	+	-	Growth	++	+	+	++
OYL-025	Bavarian Wheat	+	-	Growth	++	++	+	++
OYL-027	Belgian Saison I	+	-	Growth	+++	++	++	+
OYL-040	Belgian Dark Ale	+	-	Growth	+	-	-	+
OYL-042	Belgian Saison II	+	-	Growth	+++	++	++	++
OYL-055	Vermont Farmhouse	+	-	Growth	+++	++	++	+
OYL-200	Tropical IPA	+	-	Growth	++	+	+	++

+++ Strong activity
++ Medium activity
+ Mild activity
- No activity
n.d. Not determined

A. PCR performed using SD-5A (5'-CAACTACGACTTCTGTCATA-3') and SD-6B (5'-GATGGTGACGCAATCACGA-3') primers (Yamauchi *et al.* 1998).
B. PCR performed using STA1_UAS_Fw (5'-CCTGGCTCAAATTAAACTTTCG-3') and STA1_UAS_Re (5'ACCACCAATAGGCAATAGAAA-3') primers (Krogerus et al. 2019).
C. Results of assays developed/tested in this manuscript. LCSM (Omega-optimized LCSM), Method #1 (Monitoring pH as a Readout of Maltodextrin-Supported Growth), Method #2 (Quantification of Dextrin Type III Degradation Using Iodine Staining), Method #3 (Maltodextrin Cross-feeding Assay to Detect Diastatic Activity).