Development of Fluorometric Assay for the Determination of β-glucosidase Activity in Brewing Yeasts

by
Andrew Molitor

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented May 14th, 2015
Commencement June 2015

AN ABSTRACT OF THE THESIS OF

Andrew Molitor for the degree of Honors Baccalaureate of Science in Microbiology presented on May 14th, 2015. Title: Development of Fluorometric Assay for the Determination of β-glucosidase Activity in Brewing Yeasts.

Abstract approved:

_____

Thomas Shellhammer

Glycosides are natural molecules found in many plants and hold potential to release new flavors and aromas into alcoholic beverages, particularly beer and wine. One way to free these flavors and aromas is by enzymatic hydrolysis catalyzed by β-glucosidase. The activities of β-glucosidase are strain and species dependent in yeast as well as other organisms. This study focused on developing a rapid method for the determination of β-glucosidase activity of many brewing yeasts. Knowledge of β-glucosidase activity would allow brewers to choose different yeasts depending on their desired effect. Through examining other studies a new method was developed using the substrate 4-MUG and beer-like growth conditions to measure intra- and extracellular yeast β-glucosidase activity. 18 yeasts were measured using this method, confirming previous findings that non-Saccharomyces yeasts tend to exhibit higher enzyme activity than Saccharomyces spp.

Key Words: β-glucosidase, glycosides, yeast, fluorometry, brewing

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Andrew Molitor, Author

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-My family and friends

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INTRODUCTION

Glycosides are natural molecules found in many plants and hold potential to release new flavors and aromas into alcoholic beverages, particularly beer and wine. With the presence of an enzyme such as β-glucosidase, glycosides can be split into sugars and odor-active compounds. For this reason many in the beverage industry are curious as how to best tap into this olfactory potential. Hops (*Humulus lupulus*) are a glycoside-containing plant used in beer to add flavor and aroma[1]. In the presence of yeast it is thought that these hop glycosides can be a new source of aroma and flavor through sufficient yeast-derived enzyme activity. Knowledge of how to best use this source of untapped potential could have a significant impact on how some beers are brewed.

Glycosides

Glycosides are odorless, non-volatile molecules found in many plants. Their metabolic purpose is to store, transport, increase water solubility, and decrease toxicity of flavor compounds[2]. In general, glycosides consist of a carbohydrate bound to another functional group via a glycosidic bond. Glycosides are common flavor precursors whereby a flavor compound, or aglycone, is bound to a sugar moiety, most commonly β-D-glucose. When cleaved apart either by acid hydrolysis or an enzyme such as 1,4-β-glucosidase, the freed aglycone is able to contribute to flavor and aroma[2]. Other sugar moieties forming glycosides most commonly include monosaccharides α-L-arabinofuranose, α-L-arabinopyranose, α-L-rhamnopyranose, β-D-xylopyranose, and various di- and trisaccharides[3,4]. Glycosides are found in grapes,

hops, malt, spices, various flowers, and many other plant products used in both brewing beer and making wine[2]. In hops specifically, aglycones with potential to introduce green mushroom flavor (aliphatic alcohol), floral, citrus, and rose flavors (monoterpene alcohols and C_{13}-norisoprenoids), and minty, wintergreen, and almond flavors (methyl salicylate and benzyl alcohol) have been found among many others[2]. However it should be noted that not all aglycones are odor-active, nor are all the odor-active aglycones desirable. Some examples of these are shown in Figure 1.

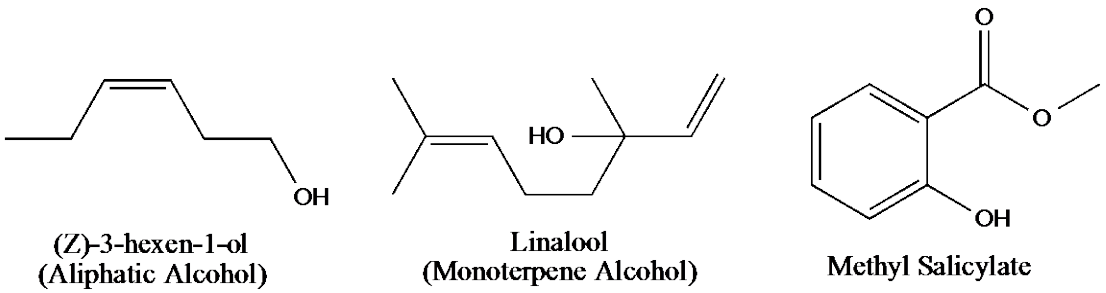

Figure 1: Examples of aglycones found in hops.

Glycoside content varies between hop varieties[5] and though little is currently known of the total glycoside concentration in hops[2], knowledge of their effective hydrolysis may drive more research in this field. The most probable way to cleave and utilize these glycosides in beer is to ferment using a yeast that produces high quantities of the enzyme β-glucosidase[6]. Acid hydrolysis, dependent on medium temperature and pH, is another means of freeing aglycones from glycosides and is structure-dependent on the aglycone, hydrolyzing certain compounds before others[3]. However, because of the acidic conditions required for this method it is not of much use in the slightly too alkaline pH of brewing.

β-glucosidase

β-glucosidase is a general term for enzymes that cleave the β bond of glucose in glycosides. β-glucosidase can come from a variety of sources including plants, bacteria, fungi, and animals[7]. For many studies examining the enzyme, this included, pure β-glucosidase purchased from a chemical supplier is isolated from almonds[8,9]. While all β-glucosidase cleaves these β bonds, the specificity, or attraction, of the enzyme to particular aglycones is variable depending on the source of the enzyme, including variation between yeast strains[9–11]. The less related two organisms are, the more likely that β-glucosidase from each will be slightly different in shape due to variation in amino acid sequence. While these differences aren't enough to alter the general enzyme function, they do have an effect on which glycosides the enzyme is most attracted to.

In a study on ale and lager brewing yeasts, β-glucosidase was shown to have an approximate molecular weight of 50,000 and function best between pH 4-6[12]. It should be noted that the molecular weight of β-glucosidase from yeast is not constant. Another study looking at *Candida* spp. found β-glucosidase to have a weight of 130,000[13], demonstrating the potentially large differences between β-glucosidases. Some research has indicated that non-*Saccharomyces* species, particularly *Brettanomyces* spp., demonstrate much higher β-glucosidase activity than *Saccharomyces* strains which are most commonly used in brewing and wine making[5,6,9,14]. Some β-glucosidases inactivate and denature quickly when exposed to heat (β-glucosidases from some ale and lager yeasts were found to become completely

inactive at 60°C) though one β-glucosidase from an archaean host has been found to be stable for 85 hours at 100°C[7,12]. In the application of brewing, inactivation of β-glucosidase from yeast due to heat is not a worry as fermentation temperatures are rarely in excess of 23°C[15]. It has been shown in many yeast that β-glucosidase expression is enhanced by aerobic conditions and repressed by glucose levels of 9% w/v or higher[8–10,14]. As less than 15% of fermentable sugars in a typical wort are glucose, fructose, and sucrose[15] (and the fact that all are reduced quickly once fermentation begins), glucose inhibition is not anticipated to be an issue in brewing. It has been observed several times that in yeast, the cell-associated β-glucosidase activity (particularly in the cytosol) is significantly greater than extracellular activity[9,10,14]. This is potentially problematic for brewing applications, due to the possibility that only extracellular enzyme (enzyme that is excreted by the yeast) acts on glycosides in solution[14]. For enzyme inside the yeast to act on glycosides in a way that introduces aglycones to the beer the yeast would need to transport glycosides across its cell membrane into the cytosol, cleave the glycoside, and then pump the aglycone back out. Or, the cell might have to lyse.

β-glucosidase and Wine

The activity of this enzyme has been studied quite extensively in wine. Many glycosides are naturally found in grape juice, creating the potential for many flavor and aroma qualities with the presence of β-glucosidase. However many of these glycosides consist of an aglycone and a disaccharide which β-glucosidase is unable to act on alone, requiring the assistance of β-glycosidase before being able to free the

aglycone[11,16]. Some studies have indicated the pH of wine to be too low for β-glucosidase to exhibit an effective level of activity[11]. In fact, many wine yeasts display much lower β-glucosidase activity than beer yeasts when examined in identical conditions[12]. Due to this inherent low pH of wine, it is possible that studies examining β-glucosidase activity of yeast in wine fermentation conditions have inflated values due to acid hydrolysis of glycosides.

β-glucosidase and Beer

β-glucosidase is of great interest for brewing beer because hops, used to contribute flavor and aroma to finished beer, contain many glycosides[2]. Hops are typically added to beer at two different points in the brewing process, during the boil (known as kettle hopping) and after fermentation (dry hopping). Because yeasts show the highest β-glucosidase activity during the exponential growth phase during fermentation[10] they have the most potential to release aroma and flavor from pre-fermentation hop additions. The most plausible and economic method to introduce β-glucosidase into the brewing process is to use a yeast with high enzyme activity[6]. As previously mentioned, *Saccharomyces* spp. commonly exhibit low β-glucosidase activity, but it is possible to ferment using a mixed culture of two or more yeasts to obtain greater enzyme activity[17–19].

Methods for β-glucosidase Detection

The most frequently used substrates for yeast β-glucosidase detection in media are ρ-nitrophenyl β-D-glucopyranoside (pNPG) and 4-methylumbelliferyl β-D-glucopyranoside (4-MUG). These substrates are acted on by β-glucosidase releasing

pNP or 4-MU which is then detected by absorbance or fluorescence spectroscopy, respectively. With both substrates it is common to use an agent such as Triton X-100 to permeabilize cells for accurate measurement of cell-associated β-glucosidase activity; however, the content and activity related to a living cell are not equivalent. To mitigate this, Daenen measured the cell-associated activities of many yeasts with pNPG without permeabilizing the cells[6]. An advantage of using fluorescence spectroscopy, and one of the reasons 4-MUG was used in this study, is that fluorescence is very sensitive with detection limits up to three orders of magnitude lower than absorption spectroscopy[20]. This increased sensitivity is due to the principles of measurement employed for each method. Fluorescence spectroscopy measures emitted light rather than the loss of light as in absorption spectroscopy. A common analogy describing this advantage is that it is much easier to see one of 10,000 lights turn on rather than one of 10,000 lights turn off. 4-MUG is known to increase response with alkaline pH as well as spontaneously hydrolyze to 4-MU in pH range of 6-7[21] causing false positives. This is not a problem for the approximate pH 5 beer fermentation conditions.

In this study a method was developed to quickly and accurately determine the varying β-glucosidase activities of different metabolically active brewing yeasts. Eighteen yeasts were examined to test the method. This assay will allow many more yeasts to have their β-glucosidase quantified leading to insight of which species can be utilized in brewing, releasing flavors and aromas previously inactive and inaccessible.

METHOD DEVELOPMENT AND METHODS

Yeast Strains

Yeasts were received from the Verstrepen lab group at the VIB lab for Systems Biology at the University of Leuven on YPD slants and stored in a cooler at 4° C. Listed below in Table 1 are the yeasts analyzed and their respective points of origin.

Reference	Identification	Origin
3001	S. cerevisiae	Wine
3002	S. cerevisiae	Ale
3003	S. cerevisiae	Ale
3004	S. cerevisiae	Wine
3005	S. cerevisiae	Ale
3007	S. cerevisiae	Wine
3008	S. cerevisiae	Ale
3009	Wickerhamomyces anomalus	Ale
3010	Candida versatilis	Cucumber brine
3011	Debaryomyces nepalensis	Sake
3012	S. pastorianus	Lager
3013	S. cerevisiae	Ale
3014	S. cerevisiae	Ale
3015	Dekkera anomala	Ale
3016	Scheffersomyces stipites	Insect
3017	Dekkera anomala	Ale
3018	Dekkera bruxellensis	Ale
3019	Kluyveromyces marxianus	Figs

Table 1: Studied yeast strains and their respective lab reference numbers and points of origin.

Once received, each yeast was grown on two slants, one to store long term in mineral oil and one to work from. All slants were stored in a cooler at 10°C.

Reagents

Maltose, glucose, disodium phosphate, sodium carbonate, and 4-Methylumbelliferyl β-D-glucopyranoside were purchased from Sigma-Aldrich. Anhydrous citric acid was purchased from EM Science. Peptone and yeast extract were purchased from Fischer Scientific. Molecular biology grade agar was purchased

from Teknova. β-glucosidase (12 U/mg solid activity = 23 U/mg protein specific activity as specified by the manufacturer) purified from California sweet almonds was purchased from Calzyme Laboratories, Inc. in San Luis Obispo, CA.

Solution and Media Preparation

McIlvaine buffer was prepared by combining 0.1 M solution citric acid and 0.2 M solution of Na_2HPO_4 to pH 5. 21.2 g/L of Na_2CO_3 in sterile water adjusted for pH served as the pH 10.2 buffer. Saline was prepared with 9 g/L NaCl. Malt Yeast extract Glucose Peptone (MYPG) medium, used to propagate the yeast, consisted of 3 g/L maltose, 3 g/L yeast extract, 10 g/L glucose, and 5 g/L peptone. Yeast extract Peptone Dextrose (YPD) agar to streak the yeasts was prepared using 20 g/L peptone, 20 g/L agar, 10 g/L yeast extract, and 10 g/L glucose. Glucose was dissolved and autoclaved separately, then added to agar mix immediately before pouring plates. Buffers, saline, and media were sterilized by autoclaving at 121°C for 15 minutes before use. Unless indicated otherwise, all solutions and media were made using sterile MilliQ water. Buffers were stored at room temperature in glass bottles. Saline was stored in a glass bottle at 2°C.

β-glucosidase and 4-MUG stock solutions were made by combining 100 U (8.33 g) and 1mM amounts, respectively, into McIlvaine buffer and sterile filtering. β-glucosidase and 4-MUG solutions were frozen at -4 C in 10 ml aliquots in screw top polystyrene test tubes.

Final Method

Yeast Culturing

Each yeast was streaked for isolation onto YPD plates and incubated at 30°C for two or more days until appropriate growth was obtained. Single colonies of each yeast were used to inoculate 10 ml of MYPG in sterile disposable test tubes, aerated by vortexing for 30 seconds, and incubated for 24 hours before doubling the MYPG volume and incubating for a final 24 hours.

Sample Prep

1.5 ml of yeast suspension was pipetted into a microcentrifuge tube and centrifuged for 5 minutes at 4200 rpm. Supernatant was decanted and used for extracellular (EC) measurements. For cell-associated (CA) measurement, the remaining pellet was rinsed by suspending in 1 ml of cold sterile saline, centrifuging for 5 minutes at 4200 rpm, decanting the supernatant, and repeating. The rinsed yeast pellet was suspended in 1.5 ml sterile McIlvaine buffer. Using a multichannel pipette, CA and EC samples were dumped into troughs and transferred to four replicate wells each of a black 96-well plate at a volume of 100 µl. CA samples were also transferred into four wells on a clear 96-well plate in volumes of 200 µl for yeast quantification by optical density measurement.

Experimental Design

For calibration and standardization each black plate was loaded with four wells containing 100 µl McIlvaine buffer, four wells containing 100 µl MYPG media, four wells containing 100 µl 100 U/L β-glucosidase, and four wells containing 100 µl of

MYPG and 100 µl of 100 U/L β-glucosidase. 100 µl of 4-MUG was added to all wells before loading the plate into a Molecular Devices Gemini XPS Microplate Reader preheated to 30°C. Fluorescence of samples and standards was then measured at 0 minutes and 20 minutes at an excitation of 365 nm and an emission of 445 nm. The clear plate was loaded into a Molecular Devices SpectraMax 190 spectrophotometer and agitated before OD was measured at 605 nm.

Calibration Curve

A calibration curve of various concentrations of β-glucosidase diluted in McIlvaine buffer was developed. To account for the extracellular measurements being performed in MYPG measurements were taken at time 0 and 20 minutes of each plate's incubation and the difference of the measurements was used rather than the final fluorescence reading. Concentrations of 100, 50, 25, 10, 5, and 0 U/L β-glucosidase were used. 200 µl of each concentration was combined with 100 µl each of 1 mM 4-MUG in McIlvaine buffer, then incubated in the plate reader at 30°C for 20 minutes with readings being taken every minute. Each concentration was replicated four times in the same 96-well plate.

RESULTS

Method Development

In order to quantify enzyme activity, it was first necessary to develop a quick and precise way to quantify the amount of yeast in the MYPG suspension since one part of the total activity measurement is dependent on the amount of enzyme present which in turn would be dependent on the amount of yeast in suspension. Initially 15 ml of MYPG suspension was filtered through a preweighed filter paper, which was then dried and weighed again as described in Daenen[6]. This process proved to be slow and imprecise. Therefore the procedure was abandoned and instead an optical density method, quantifying the yeast by use of absorbance spectroscopy as used by Fia and Rosi, was adopted[9,10].

For the β-glucosidase assay itself, initial conditions most closely represented those described by Daenen (streaking yeast, inoculating liquid medium, rinsing pellet and suspending in McIlvaine buffer, incubation time) with some modifications[6]. These included using 4-MUG in place of pNPG, stepping up yeast liquid culture volume after day one in order to ensure metabolic activity, and rinsing the yeast pellet with sterile saline twice as opposed to once. It was quickly discovered that at the original concentration of 5mM 4-MUG samples were exceeding the maximum range of the plate reader so concentration was lowered to 1mM, much closer to the 0.76mM concentration of 4-MUG used by Fia in an examination of the production of β-glucosidase by wine yeasts during alcoholic fermentation[9]. Despite this change in substrate concentration readings were still high due to 4-MU fluorescence being

enhanced by the alkaline environment created by pH 10.2 buffer[21]. Concurrently, it was observed that the pH 10.2 buffer was not halting the reactions as intended. Throughout the measurement period of 20 minutes after the pH 10.2 buffer was added the fluorescence continued to climb, indicating further reaction of enzyme and substrate. Had the buffer stopped the reaction RFU measurements would be constant over time.

Because of these issues pH 10.2 buffer was eliminated from the method. Instead of using sodium bicarbonate solution to stop reactions with 4-MUG as in Fia[9], samples were incubated in real time. This allowed enzyme kinetics measurements to be taken. In the final method fluorescence readings of samples were taken at 0, 10, and 20 minutes. In addition each plate had four wells with McIlvaine Buffer and 4-MUG, four with MYPG and 4-MUG, four with β-glucosidase, MYPG, and 4-MUG, and four with β-glucosidase, McIlvaine Buffer, and 4-MUG for standardization. Calibration, blank, and sample wells were prepared in advance of reading and 4-MUG was added just prior to measurements using a 12-channel multi pipette.

In development of a calibration curve procedures described in Abnova's β-glucosidase assay kit were originally followed, using β-glucosidase concentrations of 0, 50, 100, 150, 200, and 250 U/L. Originally written for using pNPG, the same procedures were used when the switch to 4-MUG was made. Each of these concentrations were incubated with 4-MUG for 20 minutes, creating a graph similar to Figure 2 and then using the 20 minute data from each to make the curve. After repetitive attempts in creating a linear calibration curve, it was found that data was

consistently noisy and non linear above a concentration of 100 U/L β-glucosidase as can be seen in the graph below (Figure 3). It appears as though the trend for concentrations over 100 U/L is nonlinear and approaches a quadratic function. For this reason the final calibration curve consisted of measurements using 0, 5, 10, 25, 50, and 100 U/L. Most yeast in the present study had activities in this range though it should be noted that the enzyme activity values of measuring above this range are likely exaggerations of their true activity due to extrapolation of the linear calibration curve.

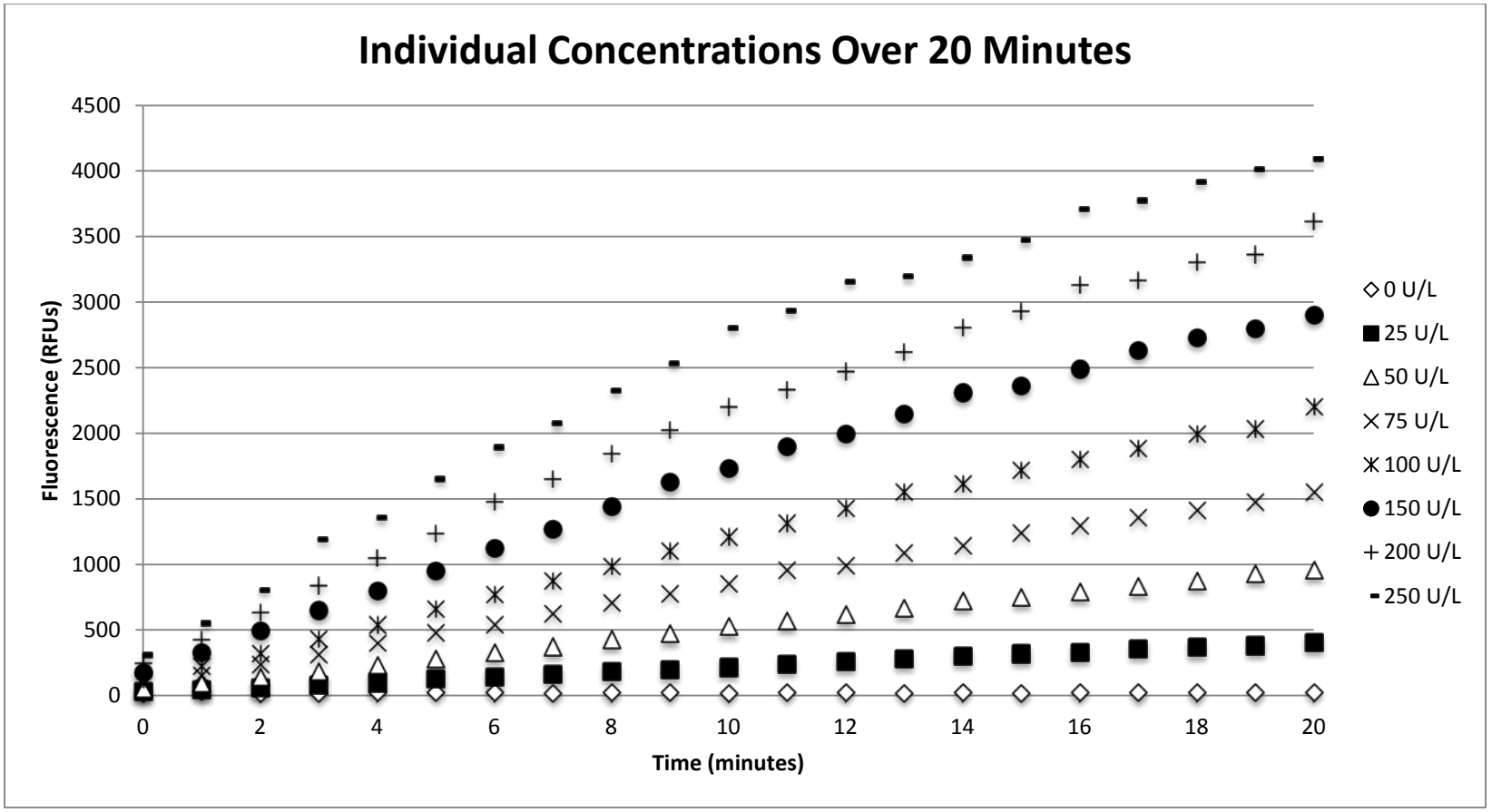

Figure 2: Individual calibration concentrations for β-glucosidase incubated in 4-MUG for 20 minutes measured once every minute.

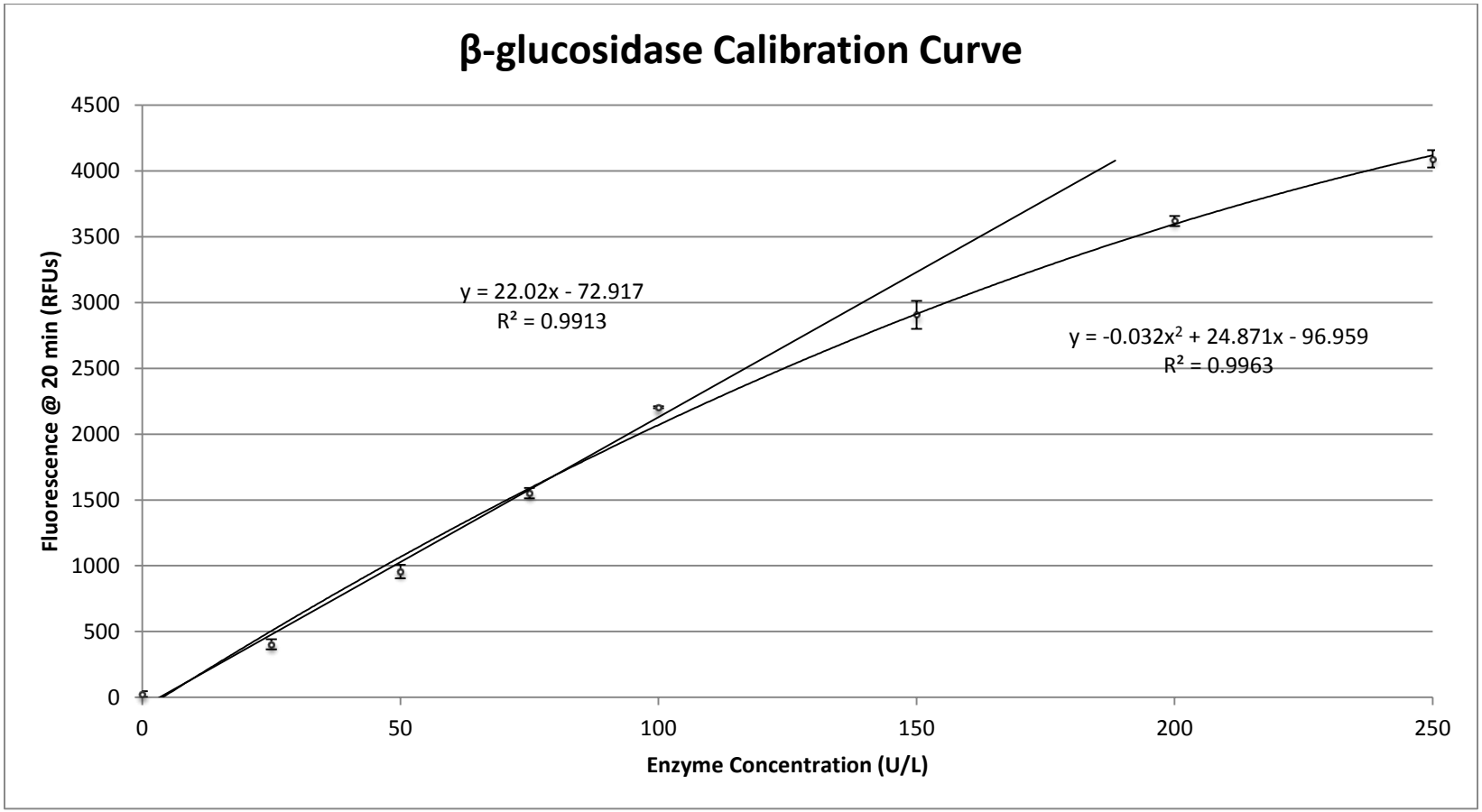

Figure 3: 250 U/L maximum β-glucosidase concentration calibration curve using 1mM 4-MUG. Error bars representing +/- one standard deviation are shown as well as a line of best fit for the concentrations at 100 U/L and below to show non-linearity in the higher concentrations.

In order to test different conditions and the possibility of storing prepared samples for up to two days a simple test was developed. Yeast 3002 was used for all method development. Factors tested were repeatability between tubes (each yeast was grown in two MYPG tubes and assayed twice), the use of 1mM 4-MUG versus 5mM 4-MUG, the use of pH 10.2 buffer stop reactions and enable incubating and storing samples for later measurement, and the storage of samples prepared to be ready for 4-MUG inoculation and incubation. Stored samples were kept in microcentrifuge tubes at 2°C.

Samples with 5mM 4-MUG appeared to give noisier and inflated readings compared to the otherwise identical 1mM 4-MUG samples (Figure 4). During method development samples incubated with 5mM 4-MUG gave too concentrated of readings for the fluorescence spectrometer on occasion, further deterring from its use in the final method.

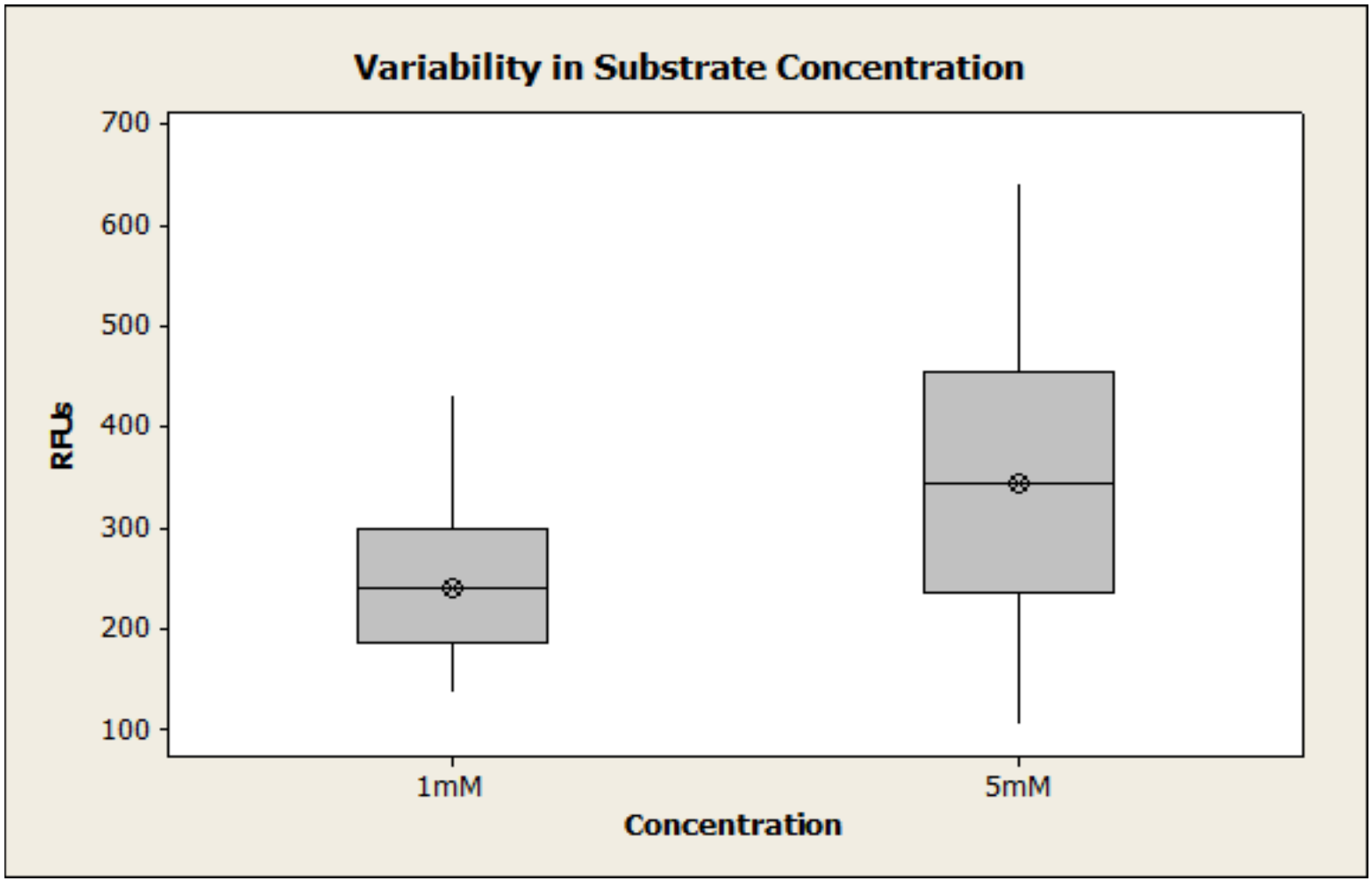

Figure 4: Boxplot comparison of the use of 1mM 4-MUG versus 5 mM 4-MUG.

Repeatability and reliability results between true tube repetitions are reflected by the boxplot below (Figure 5). A P-value of 0.447 by two-sample t-test fails to reject the null hypothesis and indicates there is no evidence to suggest a difference in mean RFU values between different test tubes of the same yeast. Statistically, the tube repetitions are serving their purpose by assuring accurate measurement of each yeast twice.

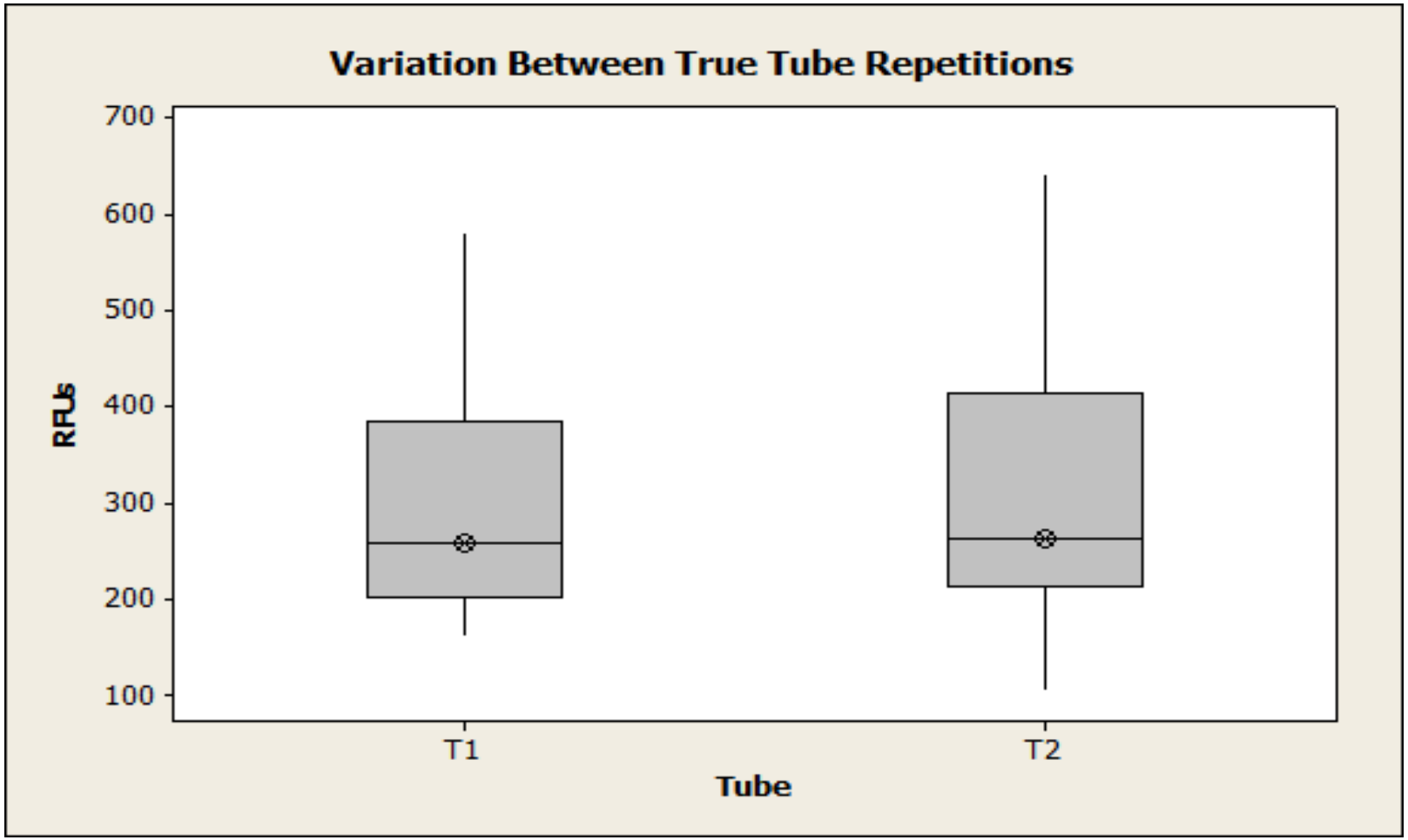

Figure 5: Boxplot comparing the reliability of tube repetitions.

To compare results between stored samples, a one-way ANOVA test was used. While there was some variation between storage times (Figure 6) the test indicated that there is no statistical difference in measurements from samples that have been stored up to 48 hours in McIlvaine buffer. Though the final method did not involve storing prepared samples for any period of time, it is feasible to do so.

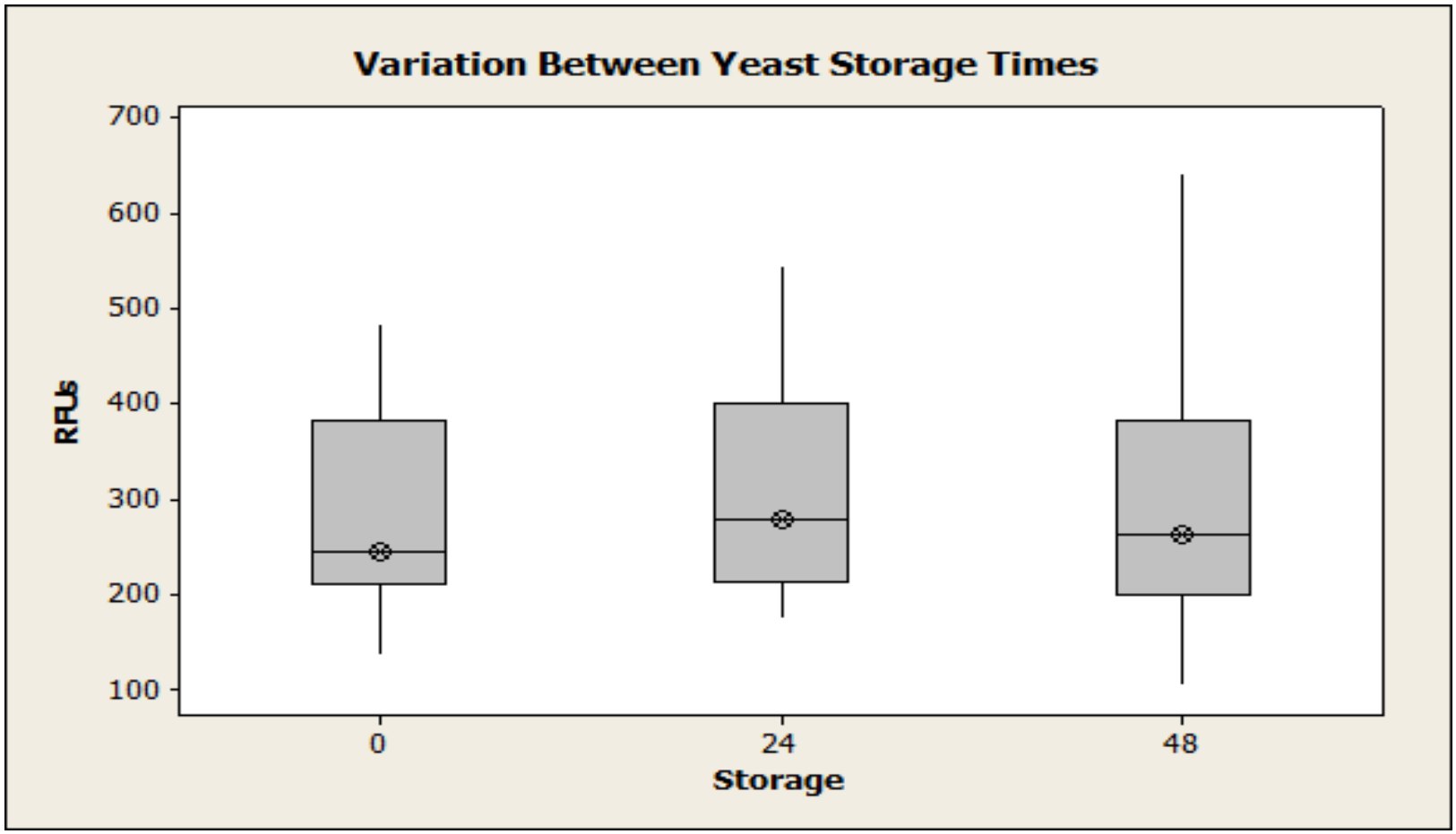

Figure 6: Boxplot comparing the fluorescence measurements of samples that have been stored for 0, 24, and 48 hours prior to 4-MUG addition.

Given a P-value of 0.092 and using a 95% confidence interval, statistical analysis by a two-sample t-test suggests that there is no difference in mean RFU values between samples that have been incubated off the plate reader and treated with pH 10.2 buffer measured at 0 and 20 minutes. But, when presented as single measurements over the incubation period (Figure 7) and in a boxplot (Figure 8), it is clear that there is an increasing trend. Due to this trend and already fairly small P-value, we predicted that if measured over a longer period of time these samples would be statistically different and as previously mentioned, it was ultimately determined that pH 10.2 did not sufficiently stop reaction of β-glucosidase with 4-MUG.

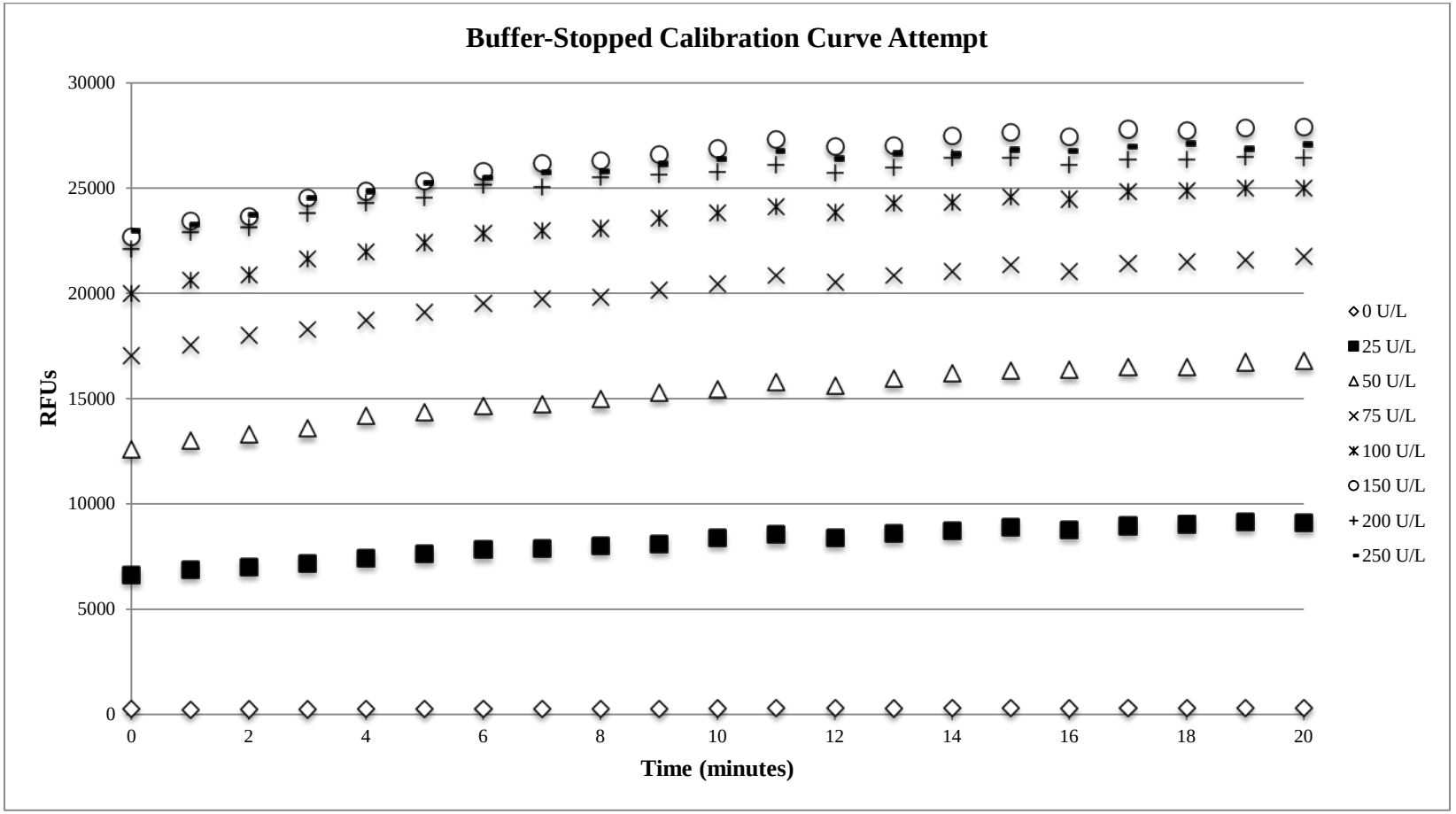

Figure 7: After incubating calibration curve samples for 20 minutes, the pH 10.2 buffer was added to each reaction. The plate was then incubated in the plate reader for 20 more minutes with readings of 365 excitation and 445 emission being taken every minute.

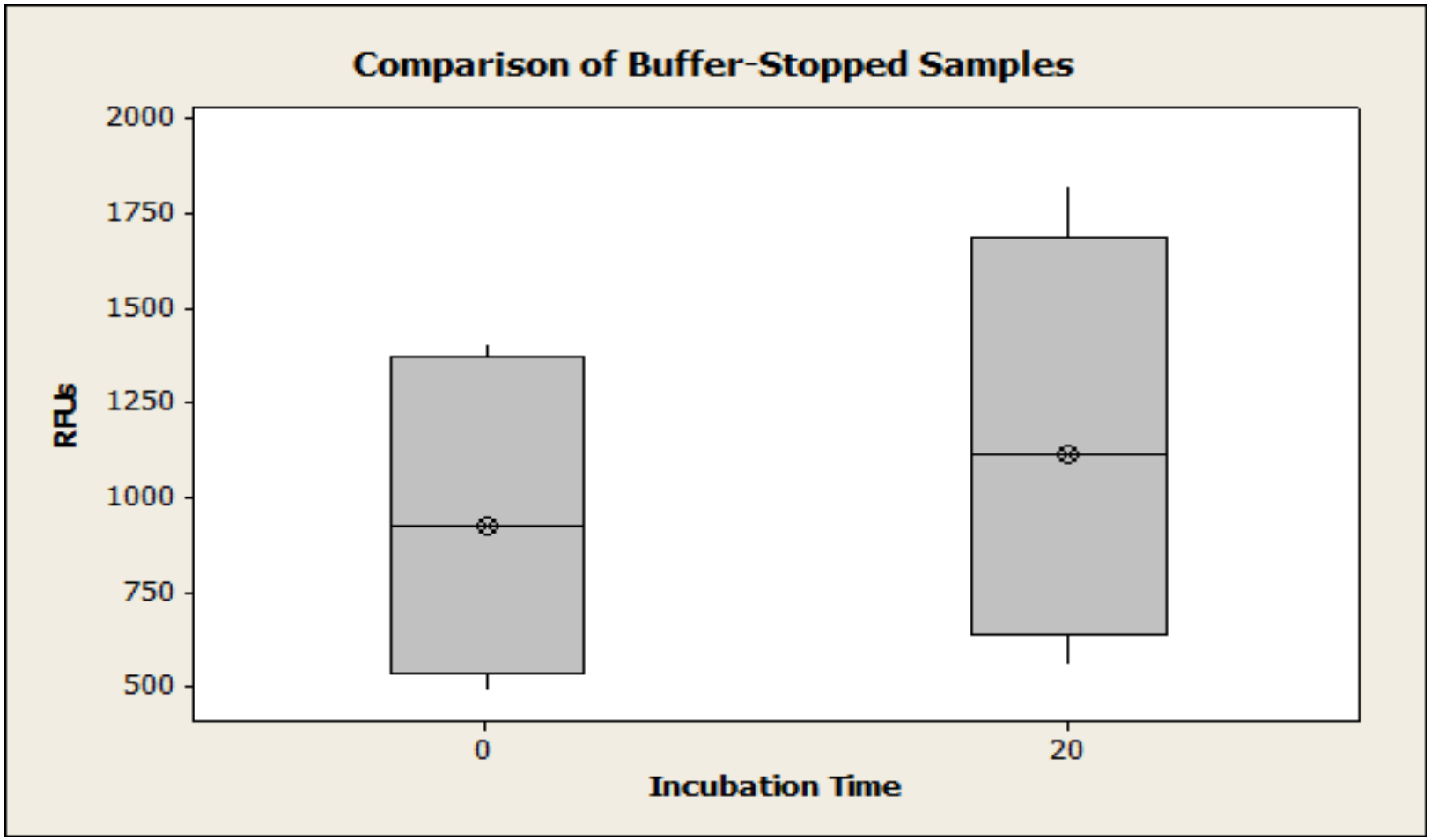

Figure 8: Boxplot of buffer-treated samples at 0 and 20 minutes. Yeast samples were incubated with 4-MUG off of the plate reader and treated with pH 10.2 buffer in an attempt to stop the reaction.

Calibration Curve

A linear projection was obtained for the final calibration curve, seen in Figure 9. Each point on the curve is the average of measured RFUs for all repetitions of that concentration. Because the curve only extends to 100 U/L β-glucosidase, measurement values of yeasts greater than 100 U/L are using extrapolations of the curve and therefore are less accurate. As indicated by the error bars, calibration curve measurements become less precise with higher enzyme concentration.

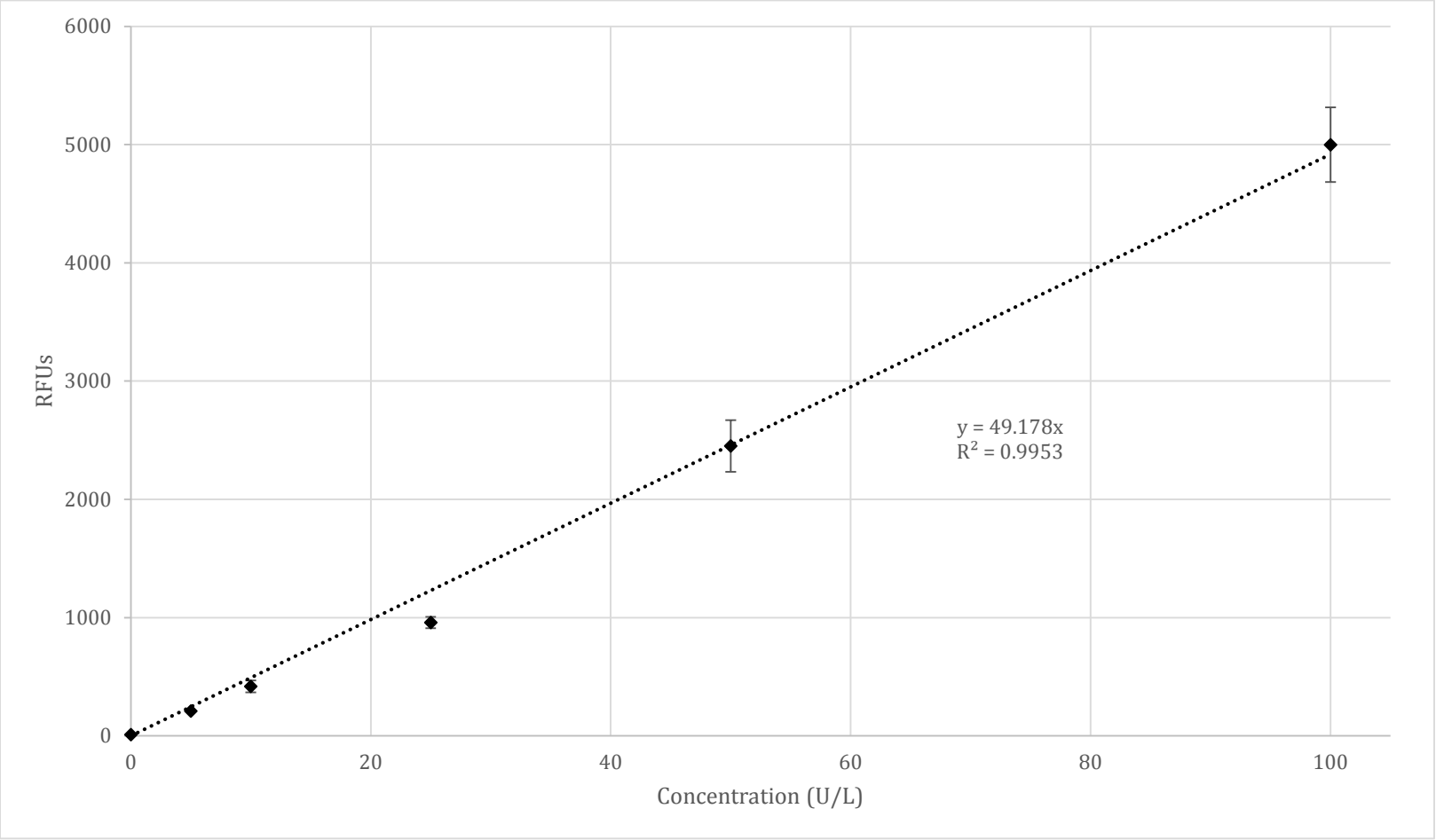

Figure 9: β-glucosidase calibration curve using enzyme concentrations of 0, 5, 10, 15, 50, and 100 U/L and 1mM 4-MUG solution. Error bars represent the standard deviation. Each point is an average of eight measurement repetitions.

In order to confirm the best emission wavelength for 4-MUG a spectrum scan (Figure 10) was carried out comparing wells containing 4-MUG and β-glucosidase in McIlvaine buffer to β-glucosidase in McIlvaine buffer (blank). The spectrum scan showed the greatest emission of 4-MU relative to the blank to be about 445 λ, as described by the product specification sheet supplied by Sigma-Aldrich.

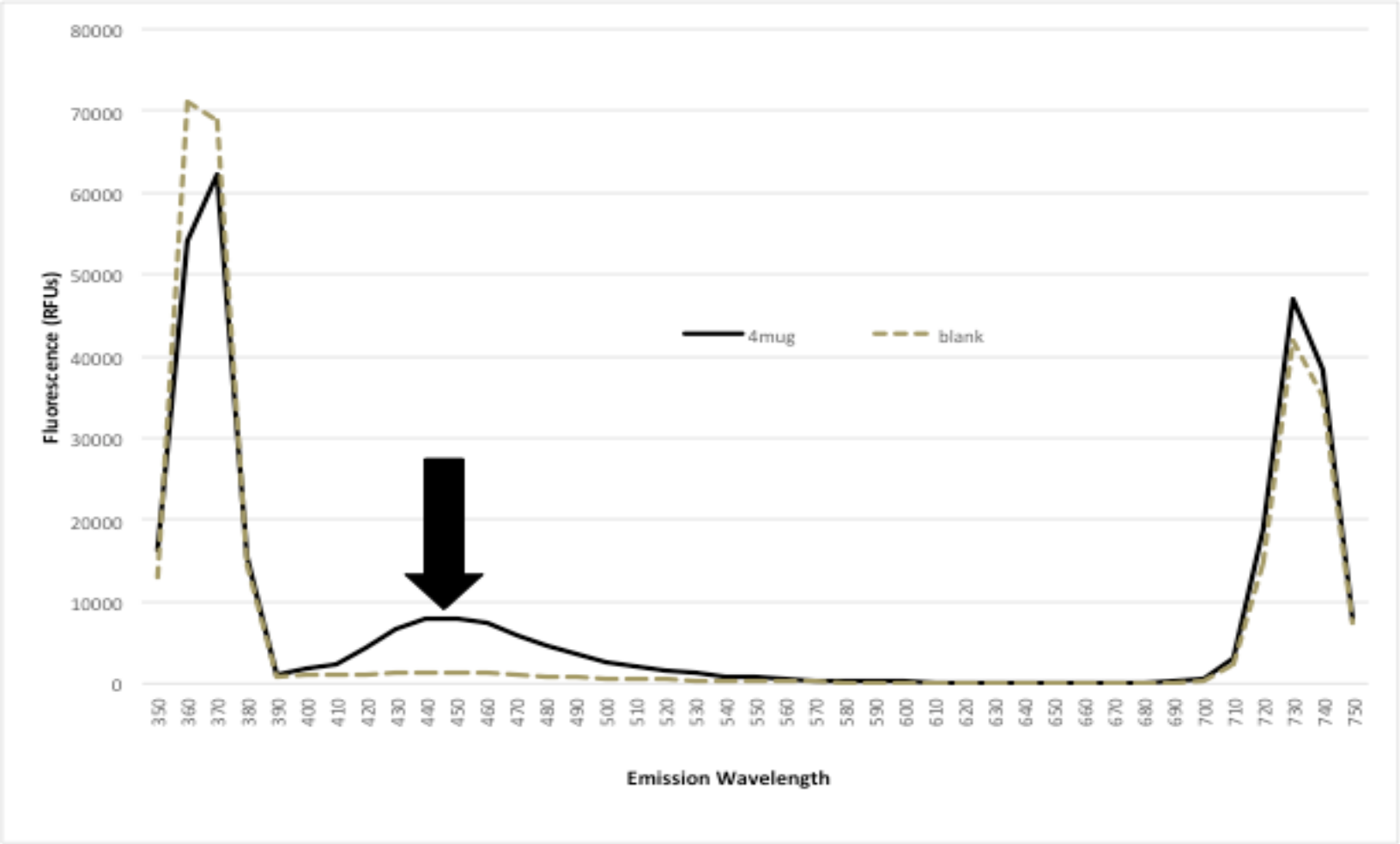

Figure 10: Spectrum scan of 4-MUG at an excitation wavelength of 365 over emission wavelengths of 350 to 750 nm.

Summary Stats/Activities

Measured β-glucosidase activities for all 18 yeast strains are presented below (Figure 11). *Wickerhamomyces anomalus*, an ale yeast, presented the largest cell-associated and extracellular activities with a combined activity of 1221 U/L_{OD605}. As the calibration curve only measured up to 100 U/L, this is an extrapolated value. *Dekkera anomala*, also an ale yeast, showed the second largest cell-associated and extracellular enzyme activities with a combined activity of 207 U/L_{OD605}. *Scheffersomyces stipitis*, *Debaryomyces nepalensis*, and *Candida versatilis*, all non-brewing yeasts, gave negative extracellular enzyme activities. When calculating the summary stats (Table 2) negative values and outliers within yeast repetitions were eliminated.

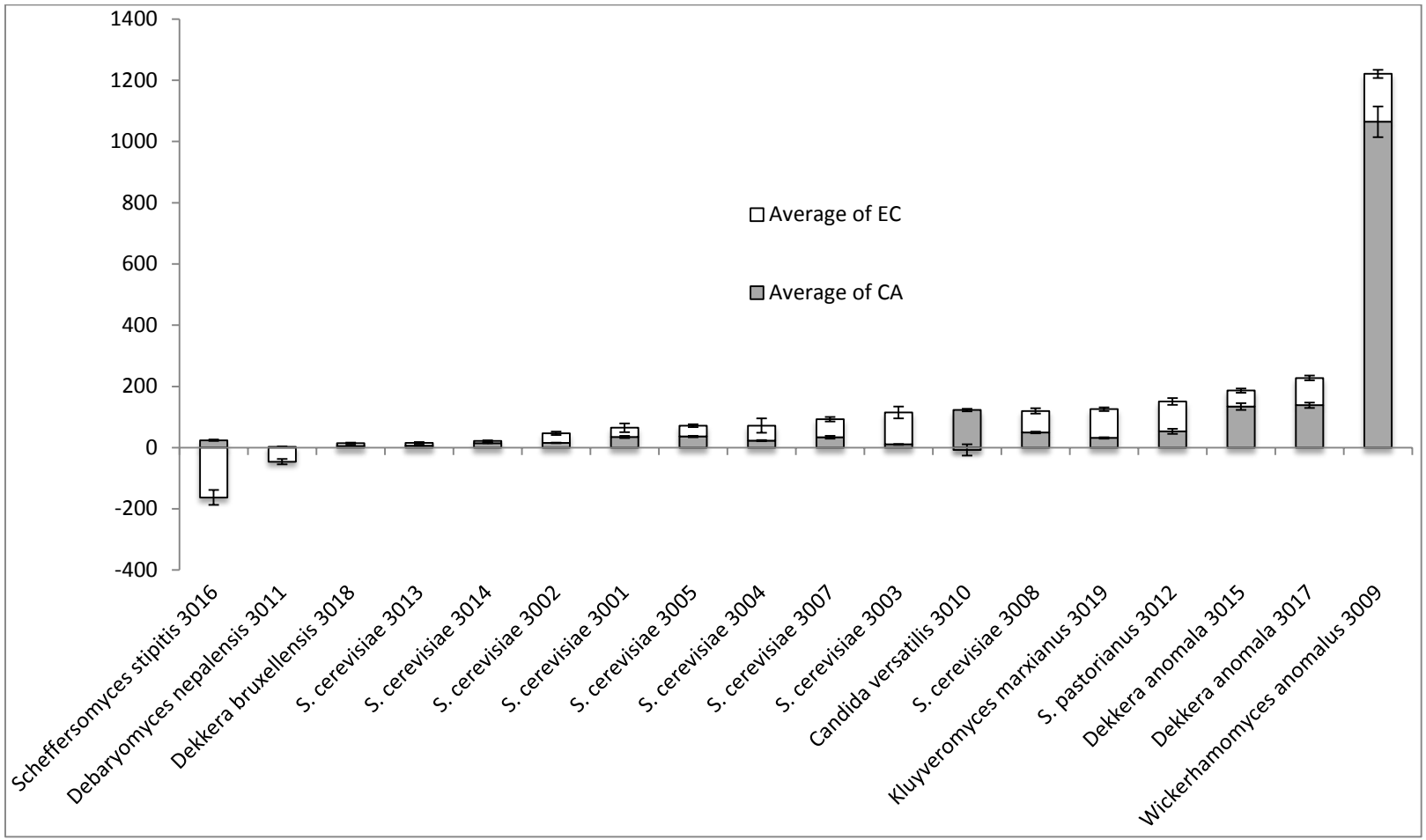

Figure 11: Bar chart representing cell-associated and extracellular β-glucosidase measurements of examined yeasts, stacked to show combined enzyme activity.

Variable	N	N*	Mean	Standard Deviation	Minimum	Median	Maximum	Range
CA U/L_{OD605}	144	0	100.1	238.7	1.0	31.5	1133.0	1132.0
EC U/L_{OD605}	113	31	60.6	42.6	4.0	52.0	173.0	169.0
Total	113	31	177.1	296.1	12.0	97.0	1291.0	1279.0

Table 2: Summary statistics for all yeasts. N indicates total number of values used for calculations while N* indicates number of values (outliers) eliminated.

DISCUSSION

The results of the yeast screening ultimately indicated that the newly developed method for β-glucosidase activity measurement was successful. The biggest obstacle in the development of this method was the creation of a calibration curve. Initial curves were based heavily off the pNPG curve created by Abnova in their commercially available β-glucosidase assay kit, using concentrations up to 250 U/L. They reportedly obtained linear results with these concentrations, but with the modifications of dissolving both substrate and enzyme in pH 5 McIlvaine buffer and using 4-MUG instead of pNPG linearity could not be obtained above concentrations of 100 U/L β-glucosidase. Fia was the only other study to provide a calibration curve, but comparison is very difficult as different enzyme quantification units were used (nmol min^{-1} ml^{-1})[9]. Most yeast displayed β-glucosidase activities well within the parameters of the calibration curve if not just slightly above for separate cell-associated and extracellular measurements with the exception of yeast 3009 (Figure 9).

A quick spectrum screening of 4-MU was carried out in order to confirm that the best emission wavelength was being used for the conditions of this assay. The scan (Figure 10) showed that 445 λ, the emission reported from Sigma-Aldrich, had the most differentiation from the blank. Emission feedback also peaked around 365 λ and 730 λ for both the blank and the 4-MUG, likely because 365 λ is the excitation wavelength and reflected back while 730 λ is twice the excitation (a second harmonic) potentially caused by refraction through the medium.

In development of the method, the first aspect considered and eventually eliminated was the use of pH 10.2 buffer to stop enzyme reaction before measurement. Many studies used a carbonate pH 10.2 buffer or other alkaline reagent to stop enzyme reaction with pNPG[6,10,12]. We found by experimental trial that pH 10.2 buffer increased 4-MU feedback (Figure 7, Figure 8). This is confirmed by Bobey[21]. Fia also used 4-MUG as a substrate and used sodium bicarbonate to stop reaction with β-glucosidase[9]. After eliminating pH 10.2 buffer from the procedure the decision was made to incubate and carry out reactions in-plate, introducing the possibility of observing enzyme kinetics.

The second aspect considered, though not thoroughly tested, was the presence of yeast during incubation and cell-associated measurement. Due to light scattering effects of yeast or other fluorescent components of the yeast it is possible that the presence of yeast during measurement could inflate fluorescence readings. In this study it was observed that the presence of yeast did increase fluorescence feedback, though this was only evaluated in the presence of pH 10.2 buffer and not for the final method conditions. As such, sufficient data was not collected and therefore none is shown in this report. There is potential that the alkaline environment of the buffer lysed the cells, releasing β-glucosidase and inflating feedback. In theory the presence of yeast should not heavily influence fluorescence measurements as the procedure detects light emitted and not absorbed. Despite this, only some studies using pNPG as a substrate removed yeast before measurements[6,12] while others opted to leave the

yeast in solution[10]. Fia, who also used 4-MUG as a substrate, took measurements with yeast present both whole and permeabilized[9].

As this study's primary focus was to create an assay analyzing β-glucosidase activity of brewing yeast for the application of brewing, enzyme activity with permeabilized cells was not examined because yeast would never be artificially permeable in practice. Fia found that β-glucosidase activity in permeabilized samples was not always higher than whole cell activity as one might expect; it was dependent on the yeast being used and the time during fermentation[9].

Concentration of substrate was variable across studies. With pNPG, both Daenen and Rosi used 5mM concentrations for β-glucosidase assay[6,10] while for 4-MUG, Fia used 0.76mM[9]. As this study was based heavily off the Daenen method initially, method development began using 5mM of 4-MUG substrate because it was assumed the rate of reaction was the same and that much substrate would be needed. Through trials it was observed that activity measurements using 5mM 4-MUG were only slightly higher than with 1mM 4-MUG (Figure 4) and in some trials with pH 10.2 buffer 5mM 4-MUG maxed out the plate reader. For this reason 1mM was used in the final method and no issues were encountered due to too little substrate.

During method development it was hypothesized that in some applications of this assay, particularly in application to assaying many more yeasts, it would be desirable to carry out yeast preparation until just before incubation with substrate and store samples so that more yeast could be prepared the next day before measuring all simultaneously. For this reason storage of samples up to 48 hours was tested. Analysis

by a one-way ANOVA test indicated that there were no differences between samples measured immediately and samples stored for up to 48 hours though due to scale and scheduling no samples were stored during this study. This result seems to indicate that β-glucosidase is stable and yeast are dormant in pH 5 McIlvaine buffer at 2°C for at least 48 hours.

In agreement with other studies, *Saccharomyces cerevisiae* yeasts measured notably lower than non-*Saccharomyces* yeasts[5,6,9,14]. Though in exception to this three yeasts measured particularly low: *Debaryomyces nepalensis*, *Scheffersomyces stipites*, and *Dekkera bruxellensis*. Two of these yeasts, *D. nepalensis* and *S. stipites*, originate from sources other than beer (sake and insect gut, respectively) and returned negative values for extracellular β-glucosidase activity. A possible reason for negative activities is a reduction in 4-MU over the incubation period due to a mechanism of the yeast and reacted 4-MUG being present at the beginning of incubation as the result of storage conditions.

The summary statistics (Table 2) lined up with other studies, showing whole cell cell-associated activities to generally be greater than extracellular activities[6,9]. It also appeared that cell-associated measurement is much more variable between yeast varieties than extracellular; the range of cell-associated activities was almost seven times as much as the range of extracellular activities. It should also be noted that some yeast appear to express different types of β-glucosidase. In a study by Leclerc et al, a *Candida* strain was found to have one parietal, extracellular β-glucosidase and one endocellular β-glucosidase expressing different specificities[13]. One possible

explanation for the difference in ranges between cell-associated and extracellular measurements is that the extracellular β-glucosidase is a more highly conserved enzyme sequence.

CONCLUSIONS

A method was successfully developed to measure cell-associated and extracellular β-glucosidase activities in brewing yeasts. During development it was noted that 4-MU fluorescence greatly increases in alkaline conditions and that 445 λ is the best emission wavelength using an excitation of 365 λ. This method was used to examine 18 different yeasts. *S. cerevisiae* consistently measured lower activities than non-*Saccharomyces* yeasts. The average cell-associated β-glucosidase activity for all yeasts in this study was almost 40 u/lod higher than the average extracellular activity. The range of cell-associated activities was almost seven times the extracellular range.

FUTURE WORK

Although it has been shown here that yeast exihibit a broad range of β-glucosidase activities, the relationship between these activites and aglycone release in beer has yet to be determined. Further investigation of β-glucosidase activity relative to the extraction rate and efficiency of glycosides throughout the brewing process would help brewers maximize the freeing of aglycones from glycosides. More research also needs to be performed on the glycoside variety and concentration in hops for brewers to be able to better predict the final attributes of their beer. Instrumental and sensory analysis of glycoside hydrolysis rates relative to β-glucosidase activities and glycoside concentrations in beer or wort would aid in determining the contribution of glycosides to aroma in finished beer.

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