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Metabolic and Morphological Evaluation of Two Brewer's Yeast Strains Throughout Serial Re-Pitching in Beer Fermentation

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Metabolic and morphological evaluation of two brewer's yeast strains throughout serial re-pitching in beer fermentation

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Table of Contents

Chapter 1: Introduction *1*

Chapter 2: Literature Review *5*

History of beer brewing **5**

Genetic diversification of brewing yeast **6**

Factors influencing yeast growth **7**

Types of brewing yeasts **9**

Homebrewing **11**

Reusing and storage of yeast in beer fermentation **12**

Programmed cell death in yeast **13**

Current microbiology testing methods for beer fermentation **14**

Chapter 3: Materials and Methods *17*

***Saccharomyces* strains** **17**

Brewing conditions **17**

Re-pitching **17**

YT plate **18**

Flow cytometry and Flocculation Assessment **19**

Viability determination assay **20**

Alcohol by volume measurement **20**

Principle Component Analysis **21**

Kruskal-Wallis test ..... Error! Bookmark not defined.

Chapter 4: Results *26*

Metabolic substrate preferences of London ale WLP013 and Czech Budejovice lager WLP802 strains after each pitch of fermentation **26**

London ale WLP013 26

Czech Budejovice lager WLP802 27

Flocculation characteristics of London ale WLP013 and Czech Budejovice lager WLP802 strains after each pitch of fermentation **27**

The viability of London ale WLP013 and Czech Budejovice lager WLP802 strains after each pitch of fermentation **28**

Alcohol by volume of London ale WLP013 and Czech Budejovice lager WLP802 strains after each pitch of fermentation **29**

Chapter 5: Discussion *40*

Metabolic substrate preferences of London ale WLP013 and Czech Budejovice lager WLP802 strains after each pitch of fermentation **41**

Flocculation characteristics of London ale WLP013 and Czech Budejovice lager WLP802 strains after each pitch of fermentation **42**

The viability percentage of London ale WLP013 and Czech Budejovice lager WLP802 strains after each pitch of fermentation **43**

Reference ***46***

Appendix ***56***

Abstract

Beer fermentation is the process of growing yeast anaerobically in a malt-based medium, ultimately resulting in alcohol and carbon dioxide production. In order to reduce the cost and waiting time, serial repitching has been practiced among microbrewers. The technique emphasizes transferring yeast from one batch of beer to the subsequent brewing cycles. It has been shown that aged yeast cultures have higher fermentation efficiency and shorter lag period, thereby resulting in shorter fermentation times, faster pH drop, and lower concentrations of unwanted products. Despite the advantages of serial repitching, one can only practice repitching for approximately 5-6 times with a good starting culture due to the decrease in brewing efficiency. In this study, we sought to further investigate the metabolic activity and morphological change of two brewer's yeast strains: London ale WLP013 and Czech Budejovice lager WLP802 throughout 8 batches of fermentation. At the end of each batch, samples were collected and subjected to YT plate and flow cytometry testing. Even though there was no significant difference in alcohol by volume throughout 8 batches, principle component analysis indicated that there were changes in metabolic potential after each batch of fermentation in the two brewer's yeast strain. Maltose, maltotriose and sucrose were common substrates correlated to these changes between strains. Czech Budejovice lager WLP802 exhibited a decrease in flocculation after eight batches, while London ale WLP013 remains at the same level of flocculation throughout eight cycles of fermentation. The findings in this study suggest that YT plate can be used as a platform to identify when brewer's yeast undergo a massive shift in metabolic potential.

Chapter 1: Introduction

Beer fermentation is the process of growing yeast on sugary water anaerobically, ultimately resulting in the production of alcohol and carbon dioxide. Since the first documentation of beer brewing was discovered in 1800 BC, brewing techniques and methods have changed and improved over the years; however, the brewing yeast, *Saccharomyces*, remains as an unchangeable factor (1). According to the Beer Institute Annual Report, U.S consumers spent $119.319 billion to purchase beer and malt-based beverages in 2017 (2).

Beer brewers initially classified fermentable *Saccharomyces* into two main groups based on their flocculation characteristics: lager yeasts are the bottom fermenters, and ale yeasts are the top fermenters. In general, the brewing process consists of four major steps: maltings and brewhouse, cellars, filtration, packaging, and distribution. In the maltings and brewhouse process, malt is produced from barley to activate the enzyme system to convert starch to fermentable carbohydrates (3). Hops and water are then added to malt to make a broth called wort. It is crucial to supply hops in this step because not only does hops give the bitterness and flavor to beer products, but it also inhibits the growth of both gram-positive and gram-negative bacteria due to the presence of iso-α-acids (4).

The cellars step is strongly yeast-related and involves yeast propagation, fermentation, handling, and maturation. Propagation describes a process in which yeast is transferred from storage to be cultured in an aerobic environment to reach the desired physiological state for fermentation. After that, propagated yeast will be introduced into wort to produce ethanol and by-products in an anaerobic environment. Temperature,

oxygen concentration, nitrogen uptake, mineral supplements, and carbohydrate ratios are the main environmental factors that will dictate the outcome of beer production (3,5,6).

Lager yeasts generally need 12 days to complete fermentation while ale yeast can take up to 14 days (7,8). After fermentation is completed, yeast cells are filtered or centrifuged from fermentation products. After that, beer is packaged and distributed to retailers.

To reduce the cost and waiting time during the propagation step, serial re-inoculation or re-pitching has been practiced among brewers in the U.S (1). This technique is employed to transfer yeast from one batch of beer to the next brewing cycles. The technique has shown to improve the viability of yeast 20-30% in comparison to the use of dried yeast usage, thereby, resulting in shorter fermentation times, faster pH drop, and reducing the risk of contamination (1,9). Despite the advantages of serial re-pitching, one can only practice re-pitching for approximately 5-6 times with a good starting culture because of the decrease in the efficiency of brewing yeast over time (10). Since it is not a precise number, homebrewers and microbrewers usually encounter problems regarding beer flavor and time required for the fermentation process to reach the desired alcohol percentage; hence, early detection of low-quality yeast can aid the business owner to decide whether the yeast can be re-pitched or not.

This phenomenon may be explained by the fact that organisms like *Saccharomyces* undergo senescence. In *Saccharomyces*, a single mother cell can reproduce approximately 25 daughter cells before it undergoes senescence and ultimately dies (11). Studies have shown that aged *Saccharomyces* cells have shorter telomere and lower telomerase activity after each replication cycle (12,13). Ijpma *et al* observed that the loss of growth potential

is correlated with the low telomerase activity and can be assayed by staining with phloxin (14). Therefore, we speculated that as *Saccharomyces* cultures age, they lose viability and can be detected using Guava Viacount Flex reagent. In addition to the physiological changes, low pH, high alcohol concentration, and toxin accumulation from the fermentation process also contribute to the decreased *Saccharomyces* fermentation efficiency due to genetic alterations (15,16).

Current viability testing relies on a variety of techniques such as cell counts, pH measurement, Gram staining, cell membrane capacitance, and flow cytometry (17). Even if the viability of *Saccharomyces* is preserved, it has been found in many cases that viable *Saccharomyces* cells do not always ferment adequately (18,19). This raises the question of whether the substrate preference, cell morphology, and cell viability percentage may change as the number of brewing pitches increases. We hypothesized that:

1. The viability and structural integrity of *Saccharomyces* strains decrease during serial repitching and stressful conditions.
2. The metabolic potential of *Saccharomyces* strains is altered during serial repitching because of the stressful environmental conditions.
3. Structural and metabolic changes can be used to predict the loss of fermentation efficacy.

The goal of this research project was to evaluate the metabolic profile and morphology of the two brewing strains of *Saccharomyces* yeasts: London ale WLP013 and Czech Budejovice lager WLP802 strains through 8 pitches of beer fermentation. Czech Budejovice lager yeast can ferment optimally from 10^{o}C to 12.8^{o}C and produce dry, crisp beer with low amounts of diacetyl production. It also has medium levels of

flocculation, 75 - 80% attenuation, and can tolerate up to 10% alcohol. In comparison to Czech Budejovice lager yeast, London ale yeast ferments in a much higher temperature range, from 20 – 23^{o}C, and produces dark malty beers. The strain also has the same levels of alcohol tolerance and flocculation as Czech Budejovice lager yeast. In terms of attenuation, the ability of London ale yeast to convert carbohydrates into alcohol and carbon dioxide is in a lower range, 67 – 75% (20,21).

In order to achieve this goal, data were generated by using i) flow cytometry to capture cellular morphology of the London ale and Czech Budejovice yeast cells after each pitch; ii) live/dead cell assay to assess the viability of the London ale and Czech Budejovice yeast cells after each fermentative batch; iii) YT plates to compare the metabolic profile of each strain at the end of each brewing session.

Chapter 2: Literature Review

History of beer brewing

The first documentation about beer brewing in human history can be found in the Hymn to Ninkasi (22). The Hymn to Ninkasi is an old-Babylonian poem, dated back to about 1800 BC, that describes basic ingredients and brewing styles of beer. According to this documentation, the Babylonian used a mixture of cooked or fermented leaves, cereal, and dried malted grains, as carbohydrate sources to feed the yeast during the fermentation process (23). During the process, honey and wine were also added to enhance flavor and the success rate of beer fermentation.

The Hebrews later learned this brewing method during their Babylon exile (24). Ancient Egyptians also described their brewing operation in greater detail on the walls of their tombs (25). Not only did they mention what raw materials and ingredients were used, but they also documented alcohol content and taste. During this period, ancient Egyptians utilized beer as a staple in their diet, for medicine, and for religious purposes. Beer brewing was then soon adopted by Europeans, who were the first to add hops to the brewing process in 822 AD (26). At the time, it was merely because hops provided the bitterness to counteract with the sweetness from the wort, but as the practice became widespread, brewers noticed that hopped beer could be kept longer. This phenomenon was explained by modern science that gram-negative bacteria, major wort spoilers, were killed by iso-α-acids released from hops (4).

Given the importance of beer in human societies, it is not surprised that beer brewing became a profession. Brewers not only mastered the technology but also

commercialized their brands and grew their markets. Beer brewing professionalism originated in central Europe, then spread all over the world. In 2004, it was estimated that Europe accounted for 34% of the world's beer (23).

Genetic diversification of brewing yeast

The brewing yeast *Saccharomyces* has been used for fermentation purposes for more than 10,000 years ago. During this period, the genetic profile of *Saccharomyces* has been changed significantly due to both artificial and natural selection (27,28). A study was published in 2019 showed that *Saccharomyces* could be divided into 13 groups based on their genetic fingerprints (29). In this study, Fay's lab sequenced 47 brewing and baking *Saccharomyces* strains, and 65 non-brewing strains. Lager, British and German ales, and a group of beer and baking strains belonged to 4 four groups, while the remaining 9 groups consisted of either common sources such as laboratories and clinics or different geographic isolations such as Asia, Europe, Mediterranean, Africa, Philippines, China, Malaysia, Japan, and North America.

Fay *et al* also suggested that, most likely, beer-brewing strains came from Asian and European populations. The conclusion was based on the results of their analysis of 64 beer-brewing strains. For these strains, the study estimated that 39.6% (ranged from 36.7% to 46.7%) of their genome was originated from the Asian population, and the remaining 60.4% (ranged from 53.3% to 63.3%) was derived from European population. They proposed a hypothesis that yeasts were hybridized from Asian and European strains.

Beer fermenting *Saccharomyces* yeasts are much more diverse in comparison to wine fermenting *Saccharomyces* yeasts. Wine yeasts are very genetically similar all around

the world, whereas beer yeasts can be very diverse (29,30). Regarding fermentation, the main difference between beer fermentation and wine fermentation is the raw materials. Wine fermentation utilizes fruits, while beer fermentation uses grains. Since wine yeasts often die during fermentation due to high levels of alcohol, serial repitching is not applied in wine fermentation; hence, it may explain the lack of genetic diversity among wine yeasts (31).

Factors influencing yeast growth

Oxygen, pH, temperature, and wort are the main factors that can affect the growth and fermentative ability of yeast; hence, these factors should be considered and carefully adjusted during yeast propagation and fermentation (13,14,15,16). Even though yeasts are classified as facultative anaerobes, oxygen remains the determining factor for yeast in fermentation because it dictates whether yeast can respire aerobically to grow or anaerobically to ferment (1). When oxygen is present, yeast utilizes oxygen to generate unsaturated fatty acids and sterols, which are used to synthesize the cell membrane (32). Studies have shown that cell membranes determine the rate of nutrient uptake and alcohol tolerance levels in yeast. Yeasts also synthesize molecules that are necessary for the catabolism of maltose, the primary carbohydrate source in the wort (13,14).

Aerating yeast in the early phase of fermentation can help to increase overall the number of yeast cells as well as individual cell mass. Studies have shown that *S. cerevisiae* grows well with oxygen concentration between 8-12 part per million (ppm), while 10-15 ppm O_2 is recommended for *S. pastorianus* (33,34,35). This poses a challenge for homebrewers because the maximum level of dissolved oxygen at atmospheric pressure in

wort is 8 ppm, and this number decreases as fermentation progresses (34). To overcome this obstacle, many industrial and microbrewers oxygenate their yeast as they pitch. With a single use of 60-second oxygenating, the levels of dissolved oxygen can reach 10-12 ppm in a 19L fermentor at atmospheric pressure (36).

Temperature also plays a critical role in the physiology of yeast. Although yeast can multiply and produce alcohols at temperatures up to 37^{o}C, it is recommended that yeast be propagated at 23-25^{o}C, and fermentation should be carried out at a much lower temperature range, 8-24^{o}C (37,38,39). High temperatures are not desirable because yeast tends to produce more esters and is less viable and stable in this environment (40). Also, growth and fermentation are exothermic processes; therefore, the temperature inside the fermentor can be higher than the outside as much as 4^{o}C.

pH is another factor that can influence yeast growth and metabolism. In general, yeast grows well between pH 4 to 6, and the starting pH of the wort is about 5.1 to 5.2 (39). This pH value can drop to 4.2 or lower by the end of the beer fermentation process. Low pH is generally beneficial for fermentation because it helps to eliminate competition from any contaminating bacteria. Brewing yeast strains can survive at pH 2.0 while many bacteria cannot, and many homebrewers utilize this difference to wash their yeast in phosphoric acid at pH 2.0 if they decide to re-use the yeast (38,39,40).

Lastly, the quality of wort dictates the performance of yeast in terms of growth and fermentation. In general, standard malt should provide enough fermentable carbohydrates for the yeast to grow and ferment; however, poor quality malt can cause underperformance in yeast due to the low levels of nitrogen, too high or too low grain protein concentration, or contamination with molds. To enhance the productivity of yeast, ammonium phosphate,

amino acids, peptides, vitamins, and zinc can be added into wort since these compounds provide additional nitrogen, and micronutrient sources for yeast (41,42,43,44). It is important to carefully consider what supplements to use because amino acids and peptides can add undesirable flavors to the final beer product.

Types of brewing yeasts

Even though both ale and lager yeasts are classified in the same genus, they display distinctive genetic and physiological characteristics. Ale yeasts are often called top fermenters, while lager yeasts are categorized as bottom fermenters due to their differences in flocculation behavior. In addition, ale yeasts prefer to ferment at higher temperatures (18-24ºC) while lager yeasts tend to perform well at a much lower temperature (8-14ºC) (1, 45).

Ale yeast, known as *Saccharomyces cerevisiae*, has been employed in the beer fermentation process since ancient Egyptian times (24). This species is also used in the bread and wine industries because it ferments rapidly due to high-temperature fermentation range, consumes the desirable set of sugars, withstands high alcohol levels and can survive the anaerobic conditions. Flavor compounds, esters and undesirable alcohols that are produced by ale yeasts can be varied. Strains that produce a low quantity of these flavor compounds are called as clean fermenters, while fruity fermenters produce a more complex profile of these flavor compounds. In the right conditions, ale yeasts will start rising to the top after the first 13 hours of fermentation and ferment for approximately 72 hours (46). This phenomenon allows homebrewers to collect ale yeast from the top or remove the foam from the top of the fermenting wort; however, in order to perform this technique aseptically, homebrewers should consider using fermenting reservoirs with large top ports.

Since ale yeast can ferment at high temperature, a small pitching density (5-10 x 10^6 cells/ml) is enough to promote cell growth and desirable beer flavor (47,48,49).

Lager yeast has never been isolated from the wild and is known as *Saccharomyces pastorianus*, a hybrid of *S. cerevisiae* and an unknown species (50); therefore, in order to preserve different strains of lager yeast, artificial propagation is continuously employed. Unlike many ale yeasts, lager yeasts produce less esters and undesirable alcohols. Cold fermentation (8-14ºC) also prevents lager yeasts from proliferating like ale yeast and lowers the ability of lager-yeast cells to absorb diacetyl due to high sulfur concentration in the environment. Lager yeast fermentation often must undergo a process called diacetyl rest. In addition to these difficulties, a higher pitching rate (15-20 x 10^6 cells/ml) is also required for lager yeast (47,48,49).

Both lager and ale yeasts are unable to produce phenolic tasting beer due to their lack of *POF1* gene that encodes for ferulic acid decarboxylase (47,48,49). Brewing yeasts are identified in laboratories using a variety of technique include colony and cellular morphologies, fermentation properties, growth temperature, and melibiose usage. Casey *et al* has proposed that transverse alternating field electrophoresis can be utilized to generate a set of chromosome data to compare and identify different yeast strains (51). The results of the study showed that most lager strains could be categorized into two groups, Carlsberg and Tuborg, while ale strains were much more diversified in their chromosome fingerprints.

Homebrewing

Homebrewing has been increased dramatically over the last two decades. Current homebrewing methods can be divided into three groups based on how the base of the beer is created. The first method is extract brewing, which involves using extracts from the grains to form wort. The extracts can be dried, liquid, or mixture of both. Extract brewing typically is less time-consuming, and requires less space and equipment, making it easier to carry out for homebrewers. The partial mash brewing method utilizes both malt extracts and grain to create a more flavor, appealing with more body beer. The third method is full-grain brewing, which is the most ancient and purest form of brewing. The process, as its name suggests, doesn't use any malt extracts; hence, all carbohydrates are extracted solely from the grains (52).

The next step of homebrewing is mashing. When grain is introduced to hot water, the high temperature will break down starches, and activate essential enzymes in the grain to convert the starches to fermentable carbohydrates (53,54,55). Higher-temperature water is also poured over the grain to wash any of the remaining sugars. Once the mashing is over, the wort is boiled at a much higher temperature over a long period of time. During this step, hops are added, and which provide bitterness to counteract the sweetness of sugars and iso-α-acids to prevent the growth of gram-negative bacteria (4).

When the boiling process is complete, the wort is cooled down as quickly as possible to prevent contamination. Among homebrewers, it is desirable to get the wort down to room temperature within 20 minutes. Depending on the size of the batch, either ice or wort chillers will be employed to achieve this goal. Yeast is added after the wort is cooled down to room temperature. Brewing yeast can come in two different forms: dry or

liquid. Since a package of dry yeast has more cell than a bag of liquid yeast, choosing dry yeast to brew beer means that brewer doesn't need to make a yeast starter. Also, dry yeast is generally less expensive and has a longer shelf life than liquid yeast (9,56). On the other hand, liquid yeast can be grown by brewers and stored for future use while many of yeast strains cannot survive the dehydration process and can only be cultured in liquid form. It is critical to aerate the wort at this step because yeast requires oxygen to grow at the early stage of the fermentation. After the fermentation process is complete, beer can be siphoned out of the fermentor and stored at room temperature for carbonation.

Reusing and storage of yeast in beer fermentation

Unlike wine fermentation, yeast can be reused in beer fermentation from pitch to pitch. It does not only save costs, but also gives brewers a jumpstart on their next batch. The use of liquid yeast has shown to improve the viability of 20-30% in comparison to dry yeast, thereby resulting in shorter fermentation times, faster pH drop, lower concentrations of diacetyl and other unwanted byproducts and reducing the risk of contamination (9). Depending on whether yeast is collected from the primary or secondary fermentor, the method can vary.

Yeast from the primary fermentor is fresher, has a higher viability, and closer to the initial culture in comparison to the yeast collected from the secondary fermentor (57,58). The process of collecting yeast from the primary fermentor can be simply described as the separation of yeast from other material in the fermentor. In general, once the beer is removed from the fermentor, the remaining particles, including yeast and hops debris, will be mixed with sterile water. After the mixture settles, three layers of liquid, yeast, and hops

debris should be observed. The liquid and yeast layers will then be transferred to a different sterile container. This process can be repeated several times to achieve a higher percentage of yeast. Yeast can be stored at 4^{o}C for later use. In addition to decanting, many homebrewers also wash their yeast with phosphoric acid, pH 2.0 – 2.5, prior to subsequent pitches (53,54).

Collecting yeast from the secondary fermentor is more straightforward than the primary fermentor due to the low amounts of hops debris in the secondary fermentor; therefore, decanting isn't necessary, and yeast cells can be collected and used right away. However, there are associated disadvantages of reusing yeast from the secondary fermentor. Yeast cells that make to the secondary fermentor have possibly diverged from the original culture, more attenuative and aggregate less than the starting pitch (59,60).

Senescence in yeast

In *Saccharomyces*, a single mother cell can reproduce approximately 25 daughter cells before it undergoes senescence and ultimately dies (11). Interestingly, these daughter cells also have the same lifespan as their mother. Studies have shown that extrachromosomal ribosomal DNA circles are often racked up in aged mother cells (12, 61). These DNA circle molecules are speculated to interfere with the governance of gene expression. Carbonylated proteins were also observed to accumulate and form aggregates in old cells (13). Although it is still unclear how extrachromosomal ribosomal DNA circles and carbonylated proteins can cause yeast cells to enter senescence or are toxic to yeast cells, both extrachromosomal ribosomal DNA circles and carbonylated proteins can be utilized as the biomarkers to indicate the age of a *Saccharomyces* cell (11).

Environmental stresses also contribute to the senescence in *Saccharomyces.* Ludovico *et al* observed that exposure to acetic acid at concentrations of 20-80 mM can trigger apoptosis in *S. cerevisiae* (62). Davidson *et al* proposed that oxidative stress can trigger cell death as well (63). The results of their study showed that cells with deleted catalase, superoxide dismutase, and cytochrome *c* peroxidase genes were more susceptible to a high heat treatment while cells with overexpression of antioxidant genes were more heat tolerant. Phenotypic changes such as disruption of the cell membrane and chromatin condensation were observed in both studies.

Current microbiology testing methods for beer fermentation

Microbiological tests in the beer fermentation consist of the use of the microscope to identify microorganisms, cell staining, cell counting, plating, ATP swabs, and polymerase chain reaction testing (17). The microscope can be the most used instrument for small-scale breweries because it is utilized for many different tasks, such as assessing the viability and vitality of yeast and estimating slurry concentration and cell density. The size of bacterial cells should be distinguishable from the yeast cells due to their difference in cellular morphology under the microscope. Also, by looking at the bottle sediments under the microscope, one can tell the microbiological composition in the brewing process.

Cell staining includes Gram and methylene blue viability stains. Gram staining helps to identify the contamination of bacteria. Gram-positive bacteria hold crystal violet – iodine complex inside their thick peptidoglycan layer and appear violet while gram-negative bacteria have a much thinner peptidoglycan layer and can be recognized as red cells (64). Gram-positive microorganisms are beer spoilers, while gram-negative bacteria

spoil wort (65). Therefore, Gram staining can be possibly used as a tool to identify contamination issues in beer brewing.

Unlike Gram staining, methylene blue viability focuses on yeast cells. Healthy yeast cells allow methylene blue to pass through their cell membrane and reduce the stain inside the cell, causing the dye to appear colorless, and dead yeast cells will stain blue. A significant problem with this method is that most dividing cells are unable to reduce methylene blue, and this can cause some confusion. Mochaba *et al* stated in their study that the method is only reliable above 90% viability (66). Methylene violet and florescent staining methods can be used instead of methylene blue viable stain, but these staining procedures require a fluorescence microscope.

Although a hemocytometer is frequently used in blood cell counting, the tool can be utilized for yeast cell counting with proper technique. Consistent dilution and pipetting correctly are the two key factors to produce accurate results with a hemocytometer because a small mistake can become a massive error in cell density. To avoid the technique sensitive aspect, more-automated approaches such as radiofrequency or turbidity can replace hemocytometer in cell counting (65).

Microbiological plating involves the use of different media to promote the growth of microorganisms in both aerobic and anaerobic conditions. Each medium has its own purpose and can be used differently to detect the presence of certain groups of organisms. For example, Universal Beer Agar is a spread plate that can be used to identify bacteria, beer spoilers, wort spoilers, and yeast spoilers, and can be incubated either aerobically or anaerobically (67). Lee's multi-differential agar is also a spread plate, but it is used mainly to detect beer, wort spoilers, and can only be incubated in aerobic conditions (68). In

addition to choosing the right type of media, the plating sample at the right time is also very important. An active fermenting sample should be analyzed within 24 hours because gram-negative bacterial cells may be lysed if the sample is held for a long period of time and hence showing a false negative.

Chapter 3: Materials and Methods

Saccharomyces strains

Both WLP802 Czech Budejovice lager yeast (*S. pastorianus*) and WLP013 London ale yeast (*S. cerevisae*) were purchased from White Labs Inc., San Diego, CA.

Brewing conditions

14.4 grams of sorghum malt extract (Midwest Supply, MN) was mixed with 100 mL of autoclaved distilled water to produce sugary water. The mixture was heated up and held at 82^{o}C for 15 minutes. Two AlphAroma hop pellets (Beer N Wine Creations, Mankato, MN) were mixed and incubated with the solution for another 15 minutes. After that, the wort solution was then cooled down to 15^{o}C in an ice bucket. In order to prevent the transfer of undissolved hops, 200 mL of cooled wort was pipetted into an autoclaved 250 mL Erlenmeyer flask. Oxygenated yeasts (5-10 x 10^6 cells/ml for ale yeast and 15-20 x 10^6 cells/ml for lager yeast) were then added to the solution, and an airlock was used to seal off the fermentor. Both stopper and airlock were sanitized by StarSan sanitizer (0.15% v/v). The temperature of fermentation was chosen based on which type of yeast being used. For example, ale yeast was incubated at 13^{o}C, while lager yeast was incubated at 23^{o}C. All fermentation reactions were incubated for ten days.

Re-pitching

After the fermentation was complete, beer was removed from the 250 ml Erlenmeyer flask by decanting, 15 ml of autoclaved water was then poured into the

fermentor and mixed. Once the solution settled, yeast and liquid layers were transferred into a sterile reservoir for subsequent usage. Gram staining and methylene blue techniques were utilized in this process to verify and check the purity of the yeast samples. Prior to a new batch, harvested yeasts from the previous batches were diluted in wort and re-inoculated into new fermentation reactions at the concentration of 5-10 x 10^6 cells/ml for ale yeast, and 15-20 x 10^6 cells/ml for lager yeast.

YT plates

YT plates were purchased from Biolog (Hayward, CA). The plate is designed for yeast identification purposes by utilizing different biochemical tests on a 96 well plate to generate a metabolic profile of the tested yeast. After harvesting the yeast cells, the cells were washed in sterile water and centrifuged for 3 times at 5000 x g. The cells were then be mixed with sterile water and diluted to a transmittance level of 47% at 490 nm (69). The cell suspension was then poured into a multichannel pipet reservoir and pipetted into YT plates (100 µl per well). The microplate contains 94 biochemical tests (35 oxidation and 59 assimilation tests) and two control wells to characterize the two brewer's yeast strains (Figure 3.1) (69). All 96 wells initially started out colorless then changed color and turbidity during incubation. These tests indicate the ability of yeast to oxidize or ferment substrates from a panel of unique carbon sources, thereby generating a metabolic profile for the tested strain.

The plates were incubated aerobically at 20°C and read at 590nm using a multiple reader MultiSkan Spectrum Thermofisher software every 12 hours for 4 days. The reading method was done using the average-well-color-development method suggested by Garland

and Mills (70). Different yeast samples have different viability resulting in either faster or slower color development, and the average well color development method helps to normalize this difference by choosing a specific time frame for each sample to analyze, so all samples are harmonized. The data were exported in Excel data sheet and standardized as follows before analysis using PC-ORD (Wild Blueberry Media LLC, Corvallis, OR). Wells that had negative value were set to zero before calculating the average well color development. The value of average well color development was the average value of 94 biochemical tests, excluding two control wells. The acceptable range for average well color development was 0.1800 ± 0.0360 for both yeast strains. Any replications that didn't reach this range were excluded from data analysis. After the data were standardized as described above, we also excluded any substrates that had zero value in all the samples before performing principle component analysis.

Flow cytometry and flocculation assessment

Yeast cells were kept cold at 4^{o}C and diluted to 250 - 500 cells/μL in 0.85% saline before subjected to the flow cytometry. The laser beam in the flow cytometry interacts with one particle at a time and differentiate different groups of cells based on how the light is scattered. Data were then be collected using Cytosoft^{TM} (EMD Milipore). In flow cytometry, a yeast population is described by two dimensions: forward scatter and side scatter. The forward scatter suggests the size of the particle, while the side scatter indicates the lumpiness.

Yeast flocculation results in big aggregates of cells. To determine the flocculation level of each brewer's yeast strain, a quad stat was applied to every sample. The threshold

of forward scatter was 2 x 10^2 FSC-HLog for both brewer's yeast strains, and any particles equal or larger than this threshold were considered as cell aggregates (Appendix 1). We combined upper and lower right quads together to calculate the flocculation level of each strain.

Viability determination assay

Yeast samples were kept at 4^{o}C and diluted to in between the range of 2.5x10^5 and 5x10^5 cells/ ml. 200 µl cell suspension was then mixed with Guava® ViaCount® Flex reagent at a ratio of 20 to 1 respectively, covered by aluminum foil, and incubated at room temperature for 20 minutes. There are two different dyes in Guava® ViaCount® Flex reagent, viability and nuclear dyes (71). The difference in permeability allows viability dye to stain dead cells and nuclear dye to stain live cells. All samples were subjected to the flowcytometry and data were collected by using the ViaCount application in CytoSoft™ (EMD Milipore).

Alcohol by volume measurement

A hydrometer was used to measure the original gravity of the wort before fermentation began, and final gravity after fermentation was completed. The alcohol by volume was calculated using the following formula: Alcohol by volume (%) = (original gravity – final gravity) x 131.25.

Principle component analysis and joint plot

Principle component analysis was conducted using PC-ORD (Wild Blueberry Media LLC, Corvallis, OR) to compare the differences of 94 different biochemical tests on the YT plate between each batch. This analytical method converses the data from the YT plate to principle components. Each principle component represents a portion of variances with the first principle component having the most amount of variance; hence, differences along the principle component 1 can be considered more important than differences along the principle component 2. The method converts the correlation among all the sample into a 2-D graph. Samples that are highly correlated cluster together. In this study, these correlations can be challenging to notice when looking at the original data in a 94-axes graph.

After principle component analysis graphs were generated, corresponding joint plots were also made using PC-ORD. In the joint plot, the inertia represents where the most common events occur, while the length of the line represents Euclidean distance value and indicates the degree to which that substrate influences the principle component analysis. The vector also indicates how the substrate respected with the principle components. A vertical vector suggests that the substrate is corresponded to the principle component on y-axis, while a horizontal vector indicates that the substrate is more corresponded to the principle component on x-axis. Diagonal vectors are corresponded to both y and x axes. In order to enhance the clarity of the joint plots , we numbered 94 different biochemical tests from 1 to 94 (Table 3.1).

Statistic

One-way ANOVA and Brown-Forsythe and Welch ANOVA tests were conducted using Prism 8 (GraphPad) to make multiple comparisons between batches in terms of viability, alcohol by volume and flocculation levels.

YT MicroPlate™

A1 Water	**A2** Acetic Acid	**A3** Formic Acid	**A4** Propionic Acid	**A5** Succinic Acid	**A6** Succinic Acid Mono-Methyl Ester	**A7** L-Aspartic Acid	**A8** L-Glutamic Acid	**A9** L-Proline	**A10** D-Gluconic Acid	**A11** Dextrin	**A12** Inulin
B1 D-Cellobiose	**B2** Gentiobiose	**B3** Maltose	**B4** Maltotriose	**B5** D-Melezitose	**B6** D-Melibiose	**B7** Palatinose	**B8** D-Raffinose	**B9** Stachyose	**B10** Sucrose	**B11** D-Trehalose	**B12** Turanose
C1 N-Acetyl-D-Glucosamine	**C2** α-D-Glucose	**C3** D-Galactose	**C4** D-Psicose	**C5** L-Sorbose	**C6** Salicin	**C7** D-Mannitol	**C8** D-Sorbitol	**C9** D-Arabitol	**C10** Xylitol	**C11** Glycerol	**C12** Tween 80
D1 Water	**D2** Fumaric Acid	**D3** L-Malic Acid	**D4** Succinic Acid Mono-Methyl Ester	**D5** Bromosuccinic Acid	**D6** L-Glutamic Acid	**D7** γ-Aminobutyric Acid	**D8** α-Ketoglutaric Acid	**D9** 2 -Keto-D-Gluconic Acid	**D10** D-Gluconic Acid	**D11** Dextrin	**D12** Inulin
E1 D-Cellobiose	**E2** Gentiobiose	**E3** Maltose	**E4** Maltotriose	**E5** D-Melezitose	**E6** D-Melibiose	**E7** Palatinose	**E8** D-Raffinose	**E9** Stachyose	**E10** Sucrose	**E11** D-Trehalose	**E12** Turanose
F1 N-Acetyl-D – Glucosamine	**F2** D-Glucosamine	**F3** α-D-Glucose	**F4** D -Galactose	**F5** D-Psicose	**F6** L-Rhamnose	**F7** L-Sorbose	**F8** α-Methyl-D-Glucoside	**F9** β-Methyl-D-Glucoside	**F10** Amygdalin	**F11** Arbutin	**F12** Salicin
G1 Maltitol	**G2** D-Mannitol	**G3** D-Sorbitol	**G4** Adonitol	**G5** D-Arabitol	**G6** Xylitol	**G7** i-Erythritol	**G8** Glycerol	**G9** Tween 80	**G10** L-Arabinose	**G11** D-Arabinose	**G12** D-Ribose
H1 D-Xylose	**H2** Succinic Acid Mono-Methyl Ester plus D-Xylose	**H3** N-Acetyl-L-Glutamic Acid plus D-Xylose	**H4** Quinic Acid plus D-Xylose	**H5** D-Glucuronic Acid plus D-Xylose	**H6** Dextrin plus D-Xylose	**H7** α-D-Lactose plus D-Xylose	**H8** D-Melibiose plus D-Xylose	**H9** D-Galactose plus D-Xylose	**H10** m-Inositol plus D-Xylose	**H11** 1,2-Propanediol plus D-Xylose	**H12** Acetoin plus D-Xylose

Oxidation Tests Assimilation Tests

Figure 3.1. The diagram of a YT plate. The 96-well plate contains 94 different biochemical tests (35 oxidation tests and 59 assimilation tests) and 2 control wells (A1 and D1). The gray wells are oxidation tests, while the white wells are assimilation tests.

1.Acetic Acid	2.Formic Acid	3.Propionic Acid	4.Succinic Acid	5.Succinic Acid Mono-Methyl Ester	6.L-Aspartic Acid
7.L-Glutamic Acid	8.L-Proline	9.D-Gluconic Acid	10.Dextrin	11.Inulin	12.D-Cellobiose
13.Gentiobiose	14.Maltose	15.Maltotriose	16.D-Melezitose	17.D-Melibiose	18.Palatinose
19.D-Raffinose	20.Stachyose	21.Sucrose	22.D-Trehalose	23.Turanose	24.N-Acetyl-D-Glucosamine
25.α-D-Glucose	26.D-Galactose	27.D-Psicose	28.L-Sorbose	29.Salicin	30.D-Mannitol
31.D-Sorbitol	32.D-Arabitol	33.Xylitol	34.Glycerol	35.Tween 80	36.Fumaric Acid
37.L-Malic Acid	38.Succinic Acid Mono-Methyl Ester	39.Bromosuccinic Acid	40.L-Glutamic Acid	41.γ-Aminobutyric Acid	42.α-Ketoglutaric Acid
43.2-Keto-D-Gluconic Acid	44.Gluconic Acid	45.Dextrin	46.Inulin	47.D-Cellobiose	48.Gentiobiose
49.Maltose	50.Maltotriose	51.D-Melezitose	52.D-Melibiose	53.Palatinose	54.D-Raffinose
55.Stachyose	56.Sucrose	57.D-Trehalose	58.Turanose	59.N-Acetyl-D-Glucosamine	60.D-Glucosamine
61.α-D-Glucose	62.D-Galactose	63.D-Psicose	64.L-Rhamnose	65.L-Sorbose	66.α-Methyl-D-Glucoside
67.β-Methyl-D-Glucoside	68.Amygdalin	69.Arbutin	70.Salicin	71.Maltitol	72.D-Mannitol
73.D-Sorbitol	74.Adonitol	75.D-Arabitol	76.Xylitol	77.i-Erythritol	78.Glycerol
79.Tween 80	80.L-Arabinose	81.D-Arabinose	82.D-Ribose	83.D-Xylose	84.Succinic Acid Mono-Methyl Ester plus D-Xylose

85. N-Acetyl-L-Glutamic Acid plus D-Xylose	86.Quinic Acid plus D-Xylose	87.D-Glucuronic Acid plus D-Xylose	88.Dextrin plus D-Xylose	89.α-D-Lactose plus D-Xylose	90.D-Melibiose plus D-Xylose
91.D-Galactose plus D-Xylose	92.m-Inositol plus D-Xylose	93.1,2-Propanediol plus D-Xylose	94.Acetoin plus D-Xylose		

Table 3.1: The numbering system of substrates on YT plate. Numbers 1-35 represent oxidation tests and numbers 36-94 represent assimilation tests.

Chapter 4: Results

Metabolic substrate preferences of London ale WLP013 and Czech Budejovice lager WLP802 strains after each fermentation

London ale WLP013

Principle component analysis of the London ale WLP013 strain indicated that the major effect in the data (i.e. separation along with the first and second principle component) was the difference between the first five batches from the rest. After the fifth batch, London ale WLP013 started to diverge from the original cluster (Figure 4.1). To further characterize the changes between batches, joint plots were generated, and their relative difference in substrate utilization was compared. In general, the joint plots suggest that there were changes in London ale WLP013 metabolic activity as the number of batches increased. The analysis of 23 samples from 93 different biochemical tests showed carbohydrates, acids and lipids were responsible for these changes (Figure 4.2).

Since maltose, maltotriose, glucose and sucrose can make up to 70% of the total carbohydrates in wort (51, 52), we compared the utilization of these carbohydrates (respectively 14,15,21,25,49,50,56 and 61) across 8 batches. Joint plots indicated that batches 1-5 had stronger utilization and oxidization of these carbohydrates than batches 6-8. In addition, batches 6-8 also had lower activity in the utilization of other complex carbohydrates (dextrin, xylose – respectively 20,83) and acids than batches 1-5 as well.

Czech Budejovice lager WLP802

Principle component analysis of the Czech Budejovice lager WLP802 strain showed that the first batch of Czech Budejovice lager WLP802 can be separated from other 7 batches (Figure 4.3), which suggesting that the metabolic potential of this strain can change as the number of batches increase. Joint plots were also generated to further understand the differences between batches. After analyzing 22 samples from 88 different substrates, we found that carbohydrates, acids, and lipids were correlated to these changes (Figure 4.4).

The joint plot indicated there were still differences in maltose, maltotriose and sucrose utilization between batches. Yeast from batches 1-5 can reduce maltose, maltotriose (respectively 49,50) better than batches 6-8, while batches 6-8 showed stronger oxidation activity for both of these substrates and glucose (respectively 14,15,25). Beside these carbohydrates, dextrin and stachyose (respectively 20, 45) were also the major drivers responsible for the differences among batches.

Flocculation characteristics of London ale WLP013 and Czech Budejovice lager WLP802 strains after each fermentation

One-way ANOVA results showed that after eight batches of fermentation, London ale WLP013 strain had a similar level of flocculation between 17.8% and 50.26% (p-value = 0.1120) (Figure 4.5). The number of aggregates was significantly lower for Czech Budejovice lager WLP802 (p-value < 0.0001) (Figure 4.6).

The levels of flocculation in Czech Budejovice lager WLP802 declined after the first batch (adjusted p-value = 0.0317); however, this loss in flocculation was restored in

second, third and fourth batch. After the fifth batch, we observed that the levels of flocculation started declining again in comparison to the first batch (adjusted p-value = 0.0153), and this trend was continuously observed until the 8th batch. At the end of the study, there was a significantly lower level of flocculation in 8th batch in comparison with 1st batch (adjusted p-value = 0.0006).

The viability of London ale WLP013 and Czech Budejovice lager WLP802 strains after each fermentation

One-way ANOVA indicated that there was a significant difference in the viability of London ale WLP013 (p-value = 0.0043) (Figure 4.7), and Czech Budejovice lager WLP802 strain (p-value < 0.0001) (Figure 4.8) after each batch of fermentation. Brown-Forsythe and Welch ANOVA tests indicated that the major difference in the viability of Czech Budejovice lager WLP802 strain is between batch 1 and a group of batches 4-8. The viability of this strain increased as number of batches increased (adjusted p-value = 0.0145 for batch 1 and 4, adjusted p-value = 0.0332 for batch 1 and 5, adjusted p-value = 0.0038 for batch 1 and 6, adjusted p-value = 0.0387 for batch 1 and 7, and adjusted p-value = 0.0400 for batch 1 and 8).

In the case of London ale WLP013 strain, the one-way ANOVA test indicated there was a significant difference among batches (p-value = 0.0043), Brown-Forsythe and Welch ANOVA tests helps to elaborate this difference more in details. The statistical results suggested that the viability started declining after batch 8. The viability of this strain decreased as number of batches increased (adjusted p-value < 0.0001 for batch 6 and 8, adjusted p-value = 0.0018 for batch 7 and 8).

Alcohol by volume of London ale WLP013 and Czech Budejovice lager WLP802 strains after each fermentation

The results of one-way ANOVA and Brown-Forsythe and Welch ANOVA tests suggest that there was no significant difference in alcohol by volume between batches in both London ale WLP013 (p-value = 0.7324) and Czech Budejovice lager WLP802 (p-value = 0.6615) strains (Figure 4.9, 4.10).

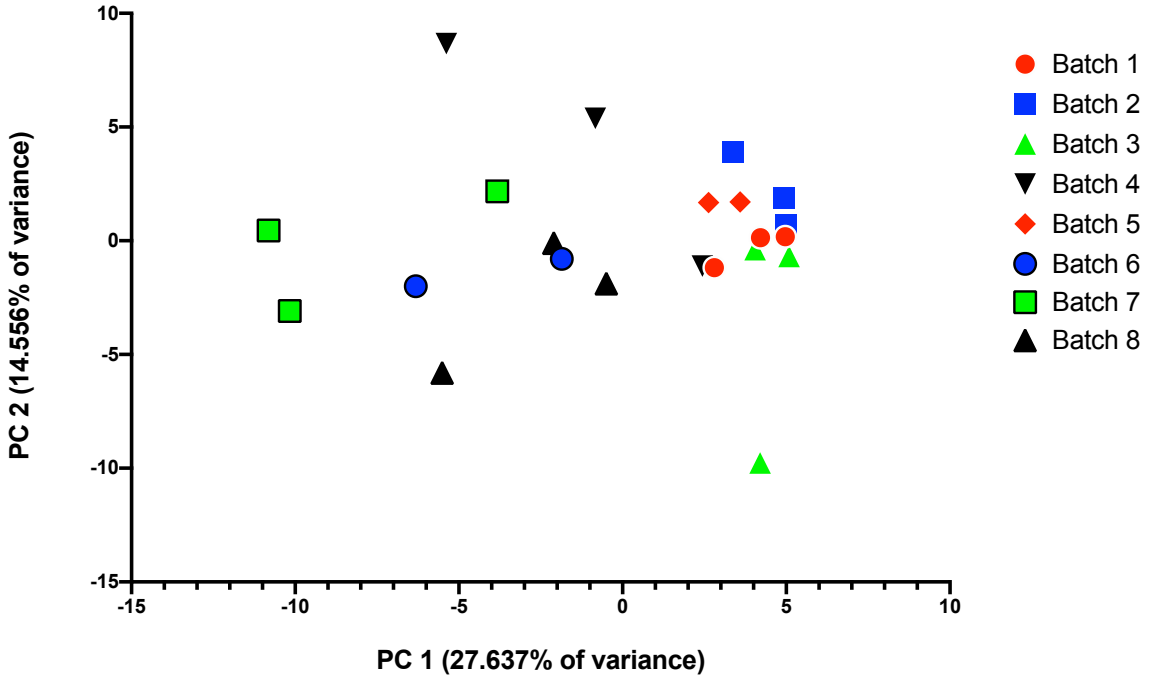

Figure 4.1. Effects of serial repitching on metabolic potential of London ale WLP013 strain. Principle component analysis (principle component 1 (27.637% of variance) and principle component 2 (14.556% of variance)) of London ale WLP013 strain from 93 different biochemical tests. The eight different batches are indicated.

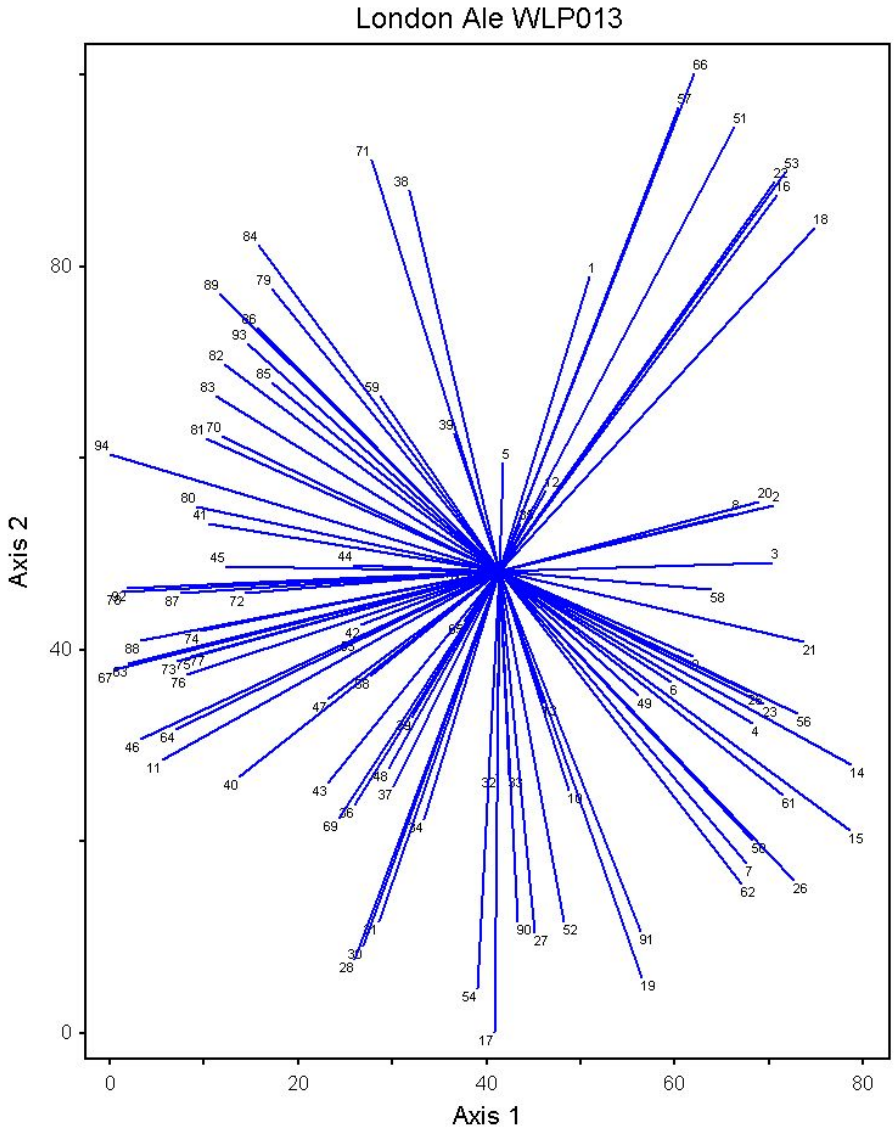

Figure 4.2. Joint plot focusing on substrates distance of London Ale WLP013 strain between axis 1 and axis 2. Substrate dots correspond to the center of the samples. The length of the line represents Euclidean distance value and indicates the degree to which that substrate influences the principle component analysis. The vector also indicates the substrate respected with principle components.

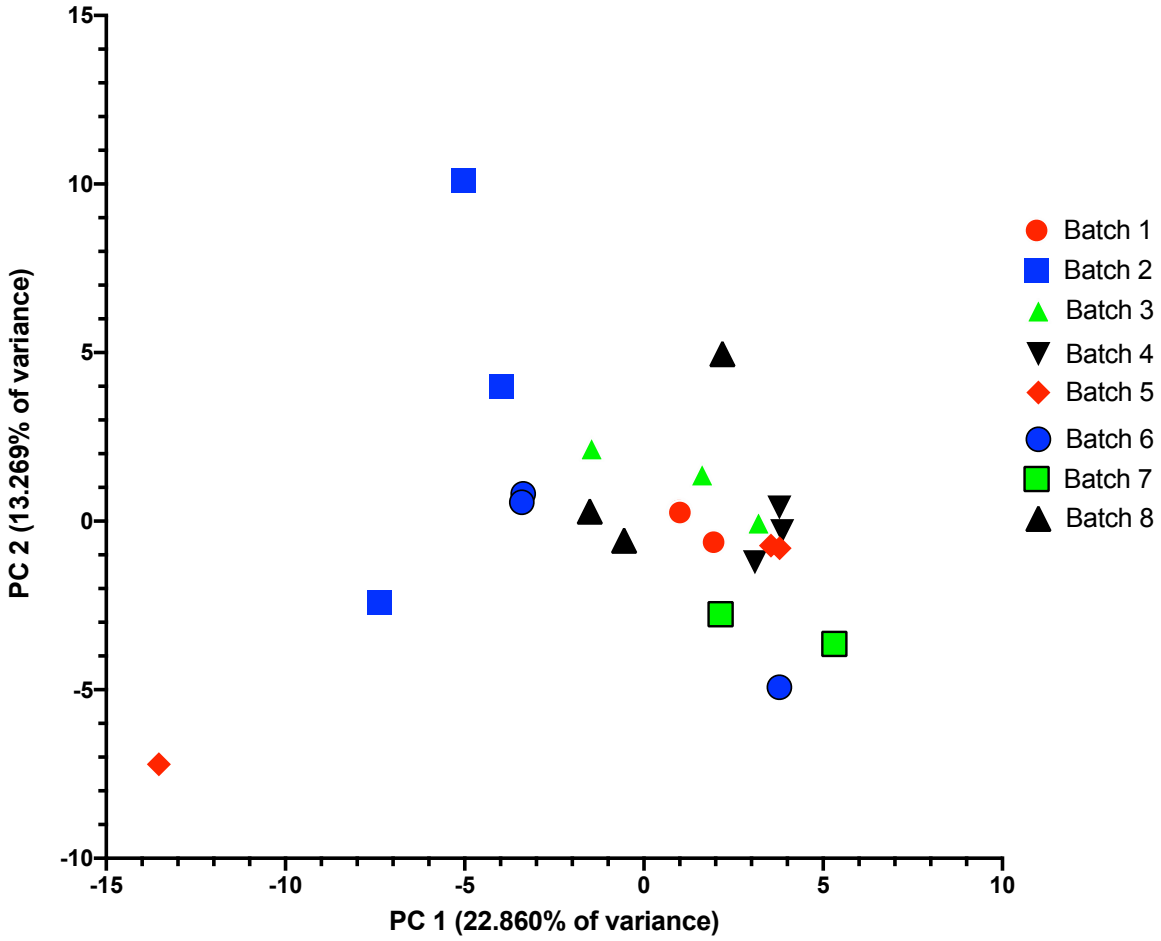

Figure 4.3. Effects of serial repitching on metabolic potential of Czech Budejovice lager WLP802 strain. Principle component analysis (principle component 1 (22.860% of variance) and principle component 2 (13.269% of variance)) of Czech Budejovice lager WLP802 strain from 88 different biochemical tests. The eight different batches are indicated.

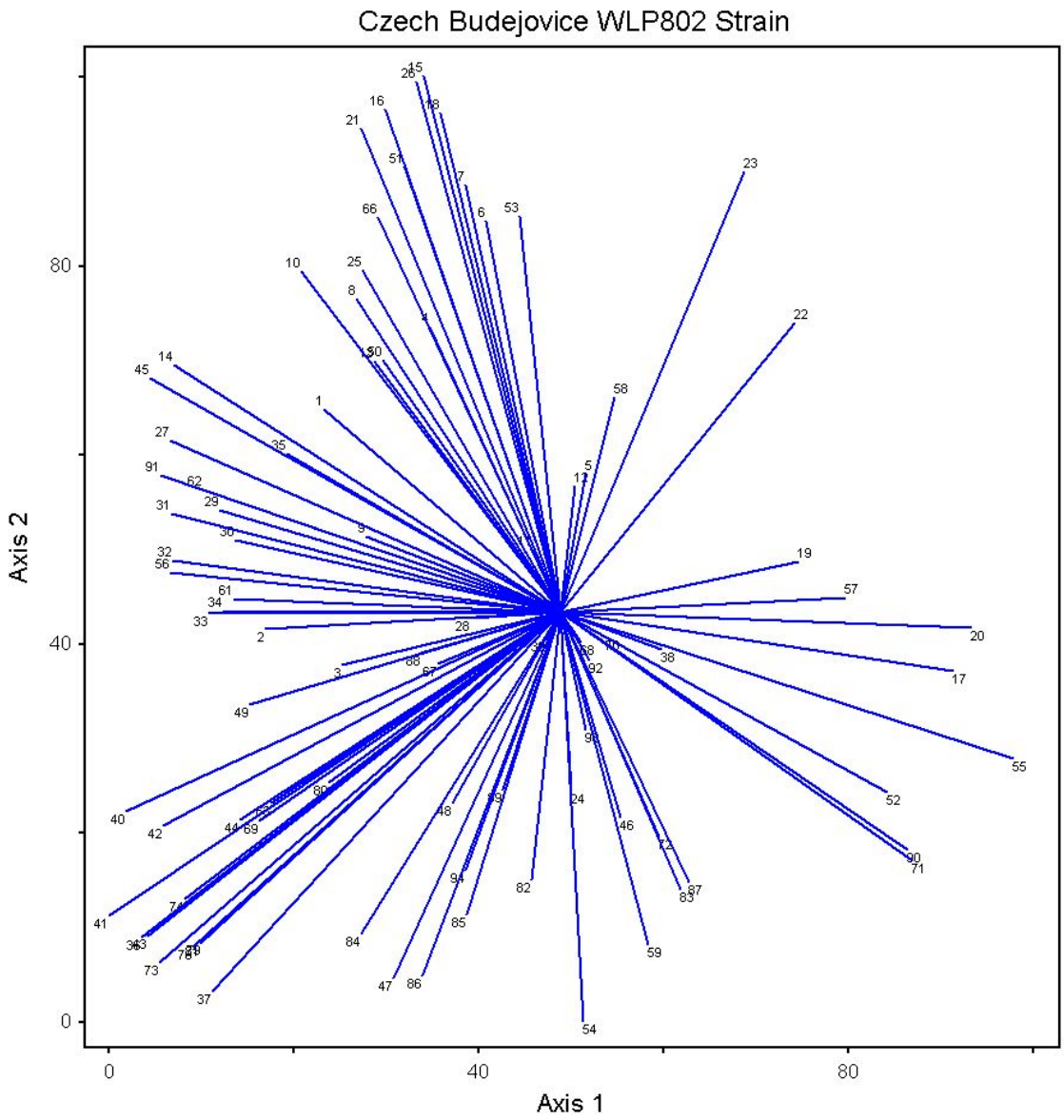

Figure 4.4. Joint plot focusing on substrates distance of Czech Budejovice lager WLP802 strain between axis 1 and axis 2. Substrate dots correspond to the center of the samples. The length of the line represents Euclidean distance value and indicates the degree to which that substrate influences the principle component analysis. The vector also indicates the substrate respected with principle components.

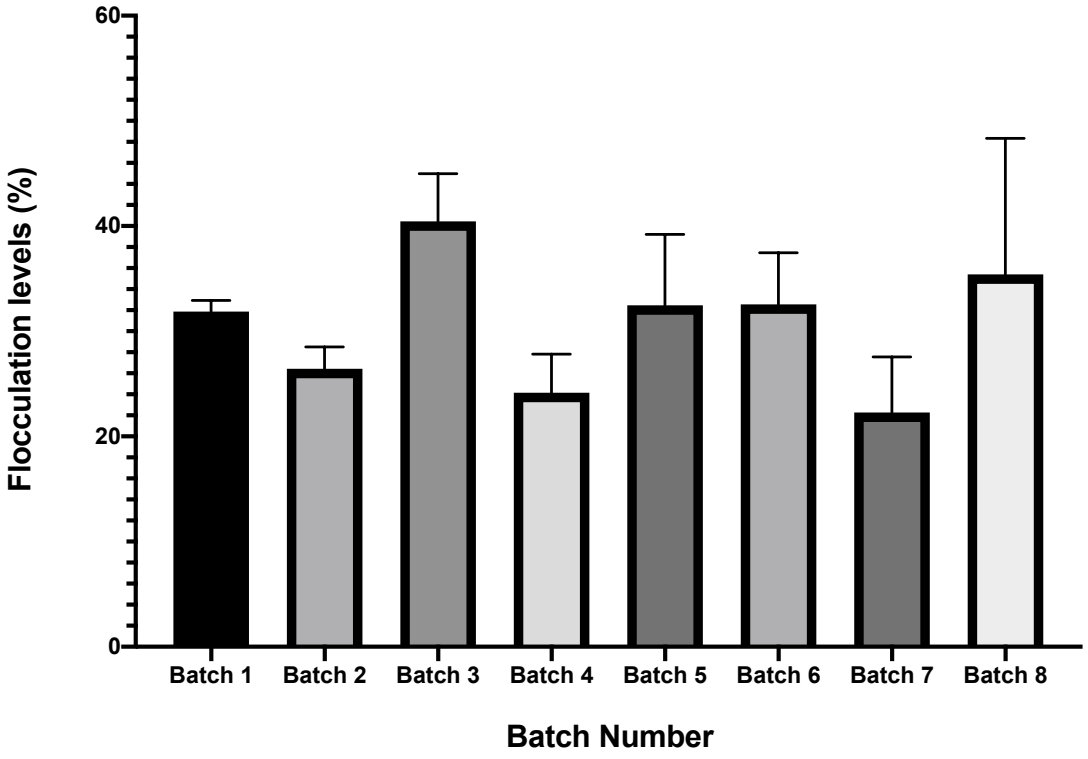

Figure 4.5. The flocculation levels of London ale WLP013 strain after each batch of fermentation. The strain maintained similar levels of flocculation throughout eight fermentation batches (one-way ANOVA p-value = 0.1120). Brown-Forsythe and Welch ANOVA tests also indicated that there was no significant difference in flocculation levels between batches.

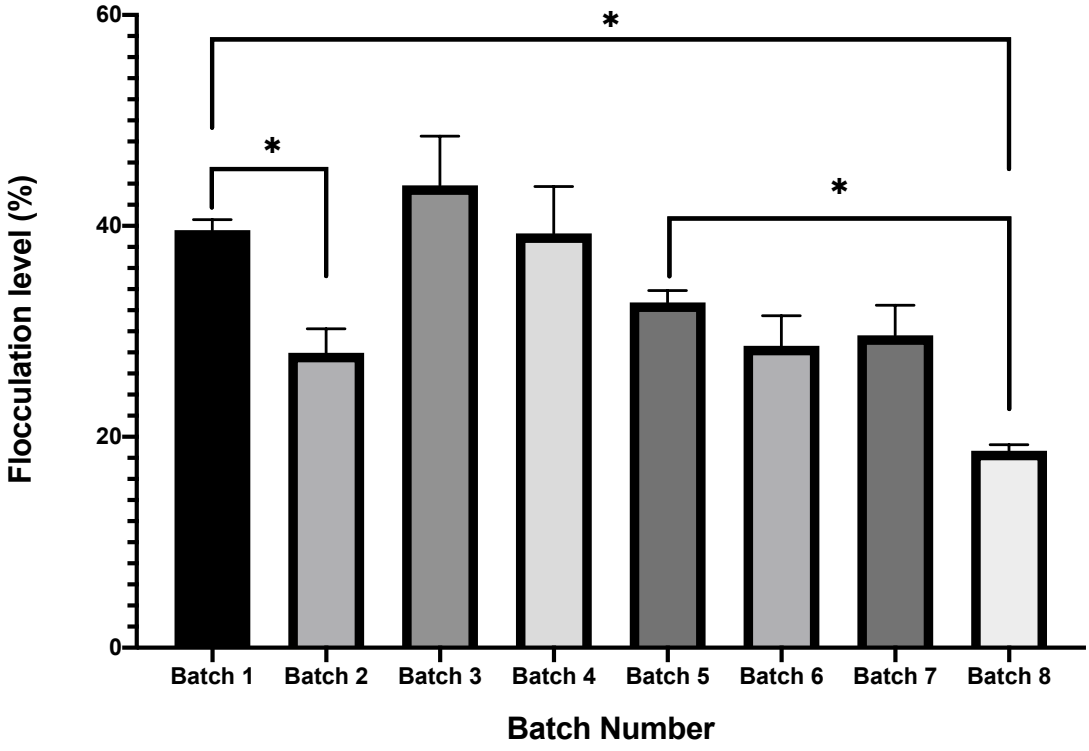

Figure 4.6. The flocculation levels of Czech Budejovice lager WLP802 strain after each batch of fermentation. One-way ANOVA indicated there was a significant difference in flocculation level (p-value < 0.0001). The 2nd batch of Czech Budejovice lager WLP802 strain had a significantly lower level of flocculation in comparison with the 1st batch (adjusted p-value = 0.0317). This loss was restored in the subsequent batches. After the 5th batch, the flocculation levels of Czech Budejovice lager WLP802 strain continuously declined to the last batch. (adjusted p-value = 0.0026 for batch 5 and 8, adjusted p-value = 0.0006 for batch 1 and 8).

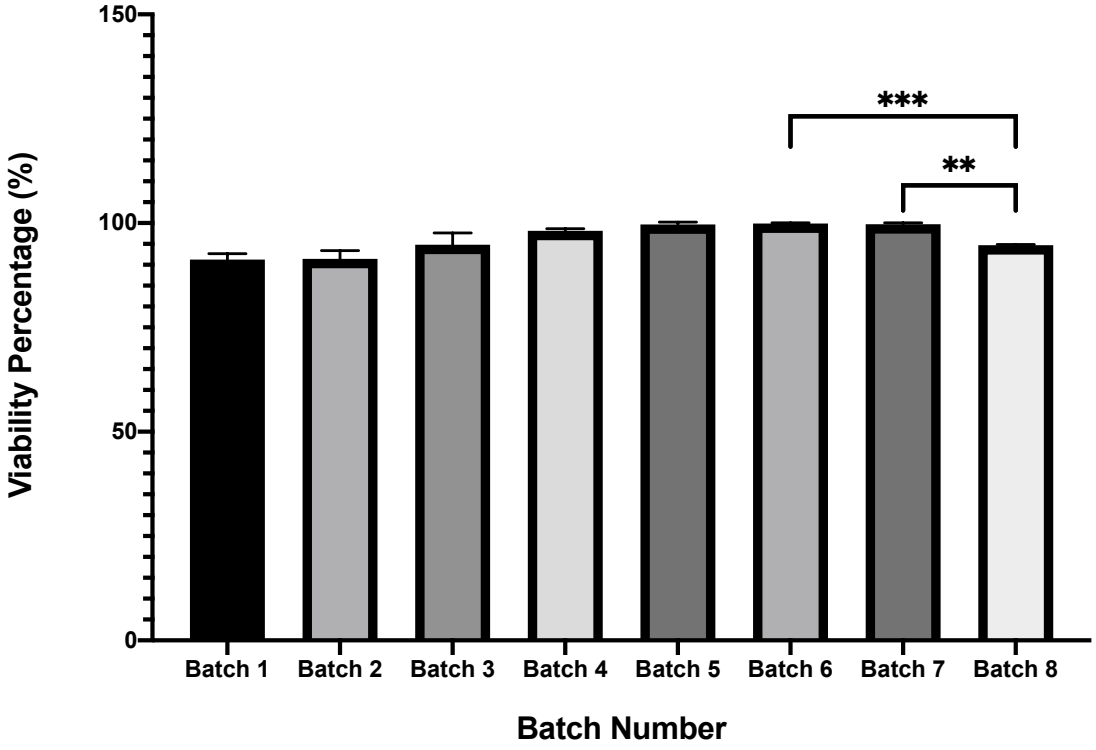

Figure 4.7. The viability percentage of London ale WLP013 strain after each batch of fermentation. One-way ANOVA suggested there was a significant difference in viability ($p=0.0043$). Brown-Forsythe and Welch ANOVA tests indicated that there was no significant difference between the first six batches, and the viability of London ale WLP013 strain dropped in the final batch in comparison with batch 6 (adjusted p-value <0.0001 for batch 6 and 8, adjusted p-value = 0.0018 for batch 7 and 8).

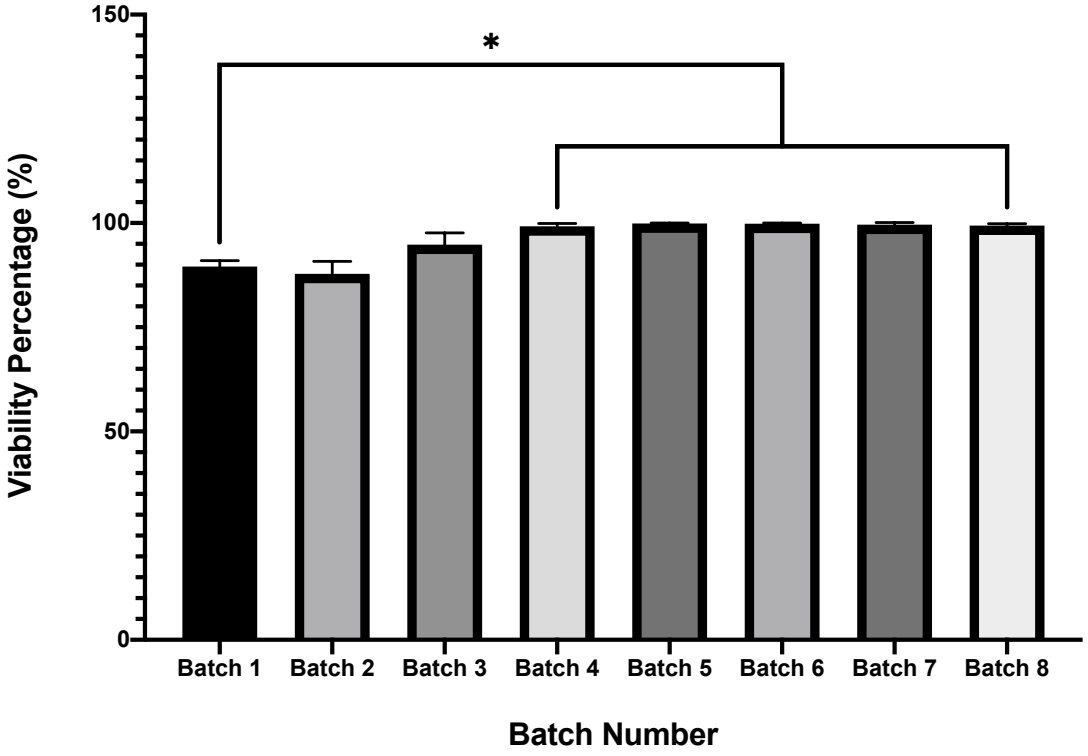

Figure 4.8. The viability percentage of Czech Budejovice lager WLP802 strain after each batch of fermentation. One-way ANOVA indicated that there were significant differences in the viability between batches (p-value < 0.0001). Brown-Forsythe and Welch ANOVA tests suggested that this difference is between batch 1 and a group batches 4-8 (adjusted p-value = 0.0145 for batch 1 and 4, adjusted p-value = 0.0332 for batch 1 and 5, adjusted p-value = 0.0038 for batch 1 and 6, adjusted p-value = 0.0387 for batch 1 and 7, and adjusted p-value = 0.0400 for batch 1 and 8).

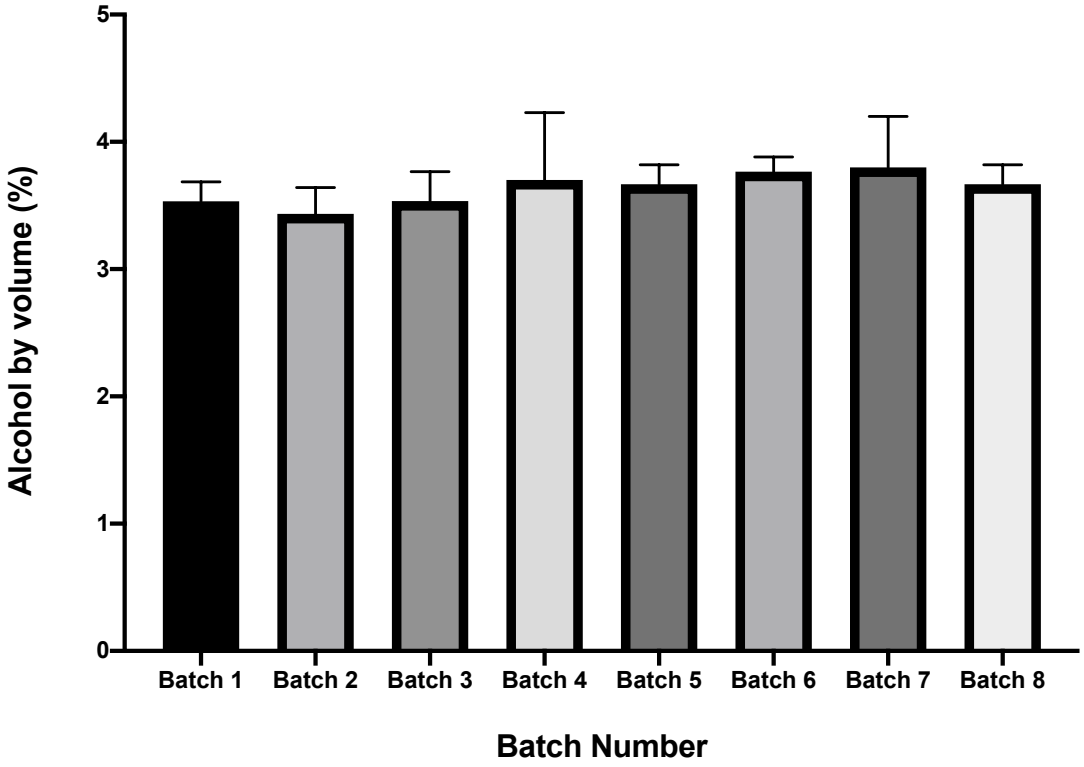

Figure 4.9. The alcohol by volume of London ale WLP013 strain after each batch of fermentation. Both one-way ANOVA and Brown-Forsythe and Welch ANOVA tests indicated that there was no significant difference in alcohol by volume between batches (p-value = 0.7324).

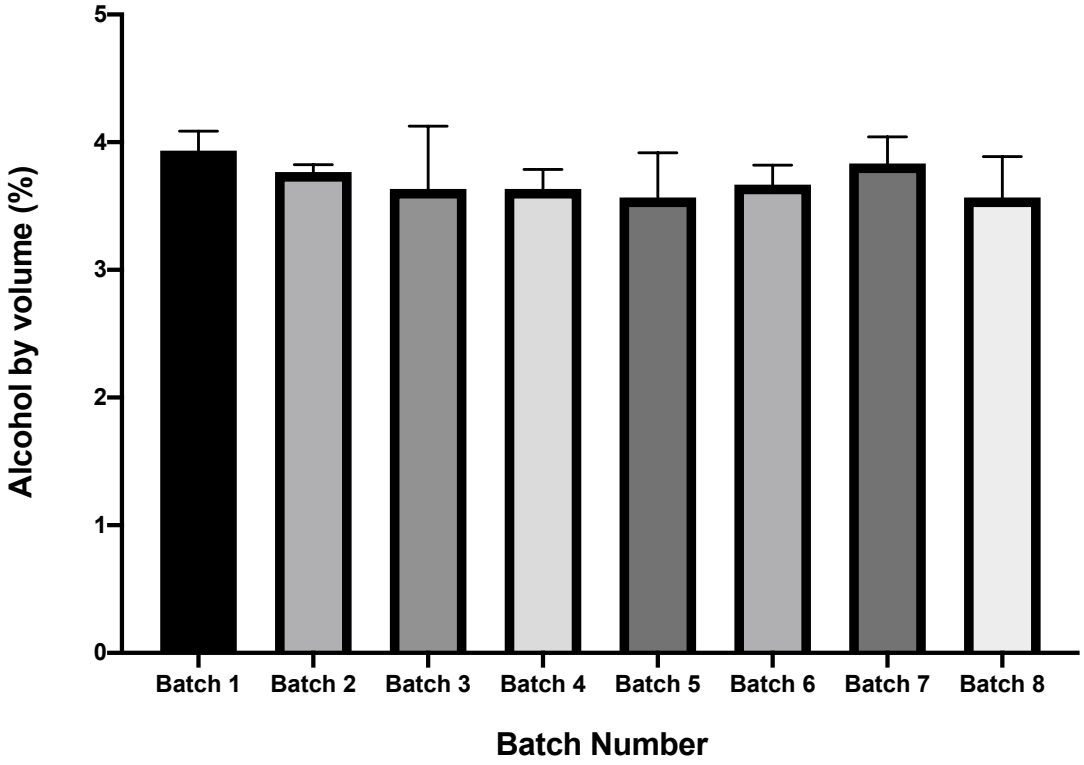

Figure 4.10. The alcohol by volume of Czech Budejovice WLP802 strain after each batch of fermentation. Both one-way ANOVA and Brown-Forsythe and Welch ANOVA tests indicated that there was no significant difference in alcohol by volume between batches (p-value = 0.6615).

Chapter 5: Discussion

Serial repitching is employed to transfer yeast from one batch of beer to the next brewing cycles. The technique has shown to improve the viability of yeast and reduce lag time, thereby resulting in shorter fermentation times, faster pH drop, and reducing the risk of contamination and undesirable products (1,9). Despite the advantages of serial-repitching, homebrewers can only practice repitching for less than 6 times with a good starting culture because of the decrease in the efficiency of brewing yeast over time (10). According to Brewer Associations, there were 2254 brewpubs and 3812 microbreweries in 2017 in the U.S. Overall, the beer market has been estimated to contribute $111.4 billion to the U.S economy and provided more than 500,000 jobs (72). It is clear that fermentation efficiency is a critical concern of the brewing industry.

The long-term goal of this research project is to develop an inexpensive and rapid quality control that can be used in any size of brewery for more consistency and profitability. This work used three different assays to analyze the flocculation levels, metabolic profile, and viability of London ale WLP013 and Czech Budejovice lager WLP802 strains through 8 pitches of beer fermentation. Each data set depicted different aspects of London ale WLP013 and Czech Budejovice lager WLP802 strains in serial repitching. The YT plate assay, in combination with principle component analysis, generated metabolic patterns that were examined for changes in substrate preference across batches. Flow cytometry was used to indicate the viability percentage of London ale WLP013 and Czech Budejovice lager WLP802 strains after each batch. Flow cytometry was also utilized to evaluate the flocculation levels of both strains after each pitch of fermentation.

Metabolic substrate preferences of London ale WLP013 and Czech Budejovice lager WLP802 strains after each fermentation

Even though the fermentative quality as measured by alcohol production by volume was consistent, the metabolic profiles of both London ale WLP013 and Czech Budejovice lager WLP013 changed during the serial repitching. Previous studies have been reported that genetic alternations can occur during fermentation (51,60). It has been estimated that it takes *S. cerevisiae* about 275 generations until mutations accumulate to be noticeable under standard laboratory conditions; however, this number drops to 40-50 generations under glucose and phosphate limitation during fermentation (73). Genetic drift may not always show immediately in the *Saccharomyces* population due to continual selective conditions of beer brewing; however, it is evident that *Saccharomyces* can change its genotype and phenotype during extended yeast recycling.

Carbohydrate substrates serve as a food source for *Saccharomyces* during fermentation; hence, the metabolism of these substrates is crucial not only for beer fermentation but also *Saccharomyces* health. Previous studies have suggested that brewing conditions might drive selection for *Saccharomyces* to metabolize wort carbohydrates more efficiently (50,59); therefore, it is reasonable to hypothesize that *Saccharomyces* metabolizes maltose, maltotriose and sucrose more efficiently as *Saccharomyces* cultures age. Powell *et al* reported that serial repitching was an artificial and continual selection for *Saccharomyces* cells that were efficient in fermentation (74). The results of their study showed that re-pitched yeast population took only 87 hours to reach the desired alcohol by volume, while virgin yeast fraction took 111 hours to reach this standard. Although there was no significant difference between virgin and aged yeast in terms of final gravity,

Powell *et al* showed that gene expression was very distinctive between virgin and aged yeast fractions, suggesting that the two populations had two different fermentation profiles.

Trevisol *et al* reported that *S. cerevisiae* metabolized trehalose as a coping mechanism against protein oxidation during fermentation (75). Cells that were deficient in the metabolism of this disaccharide showed low alcohol yield and survival rate. In the present study, trehalose utilization was maintained by both strains throughout the study. James *et al* also suggested that yeast switched its genomic expression during fermentation (76). The results showed that genes involved in transport, cell wall biogenesis, oxidative stress response, and carbohydrate degradation were upregulated, while genes involved in protein synthesis, cell cycle, DNA replication, and protein degradation were downregulated.

In conclusion, the difference in levels of utilization of maltose, maltotriose, sucrose and glucose among batches were supported by previous studies, and as such will help to contribute to the standardization in the brewing industry. YT plates can potentially serve as a platform to indicate which pitch is likely to undergo a rapid decrease in brewing quality based on the metabolic profile of the strain. Future work should approach the question from a different angle, such as looking into genetic drift or gene expression of fermentation pathways in yeast.

Flocculation characteristics of London ale WLP013 and Czech Budejovice lager WLP802 strains after each fermentation

The flocculation behavior of *Saccharomyces* is determined by the components of the cell wall, which are affected by multiple genes in *FLO* locus (77). In terms of beer

quality, flocculation potential is a desirable quality as the loss of flocculation often results in cloudy and yeasty beer. Changes in the flocculation behavior of a yeast culture are often the result of genetic drift (78,79). Given that *Saccharomyces* cells undergo many changes in cell wall composition during fermentation, we hypothesized that the flocculation levels of a strain might shift throughout the serial repitching process.

In the present study, each strain exhibited distinctive flocculation changes throughout its serial repitching process. London ale WLP013 strain maintained its flocculation levels throughout 8 batches of fermentation, while Czech Budejovice lager WLP802 lost its flocculation after batch 5. Others have observed that flocculation behavior can be very strain-dependent due to genetic diversity. Powell *et al* showed that there was no significant variation in the flocculation behavior of both BridgePort ale and lager brewing strains after 135 batches of serial repitching (60). However, in another study conducted by Powell *et al*, the results indicated that re-pitched BB11 and BB28 yeast cells increased their flocculation potential by almost 40% (74).

Even though losing or gaining flocculation potential is determined by genetics, it has been reported that this change is not correlated with a loss in viability (80,81); hence, this finding supports the results of the viability study in this project.

The viability percentage of London ale WLP013 and Czech Budejovice lager WLP802 strains after each fermentation

Viability has always been a critical aspect for homebrewers to judge whether they should continue repitching their yeast. The current method relies on methylene blue to distinguish viable and non-viable cells; however, this method is only reliable if the viability

is above 90% (66). In this study, we utilized flow cytometry to assess the viability of London ale WLP013 and Czech Budejovice lager WLP802. In this study, we found that the viability of London ale WLP013 dropped in the final batch, while the viability of Czech Budejovice lager WLP802 increased after the first batch.

Previous studies suggested that the viability of yeast can be dependent on environmental and genetical factors. Smart *et al* showed that ale yeast could increase their viability and vitality up to 100% in the first 10 batches of fermentation; however, the flocculation potential and viability became inconsistent and decreased rapidly after 24 batches of fermentation (82). In a different study, Jenkins *et al* showed that the viability of lager yeast decreased as the number of batches increased (83).

In the past, flow cytometry had been used to study physiological changes of *Saccharomyces* (66, 84, 85, 86). The findings of this study once again suggest that flow cytometry can be utilized as a platform to measure flocculation behavior and viability of yeast. It provides a rapid quality control method that can be used in large scale of brewing for more consistent, predictable and profitable.

Summary

Serial repitching is a common practice among brewers to transfer yeast from one fermentation to the next brewing cycle. Studies have shown that this technique can improve fermentation efficiency and reduce lag time in the early stage of fermentation. Despite these advantages, brewers can only utilize serial repitching for a limited number of times. Once this limit is reached, yeast progressively deteriorate or produce undesirable products. The main goal of this study was to identify when brewer's yeast decrease in brewing quality

based on the metabolic potential of the strain. In this study, we found that London ale WLP013 and Czech Budejovice WLP802 strains changed their metabolic profile significantly after five batches of fermentation. Maltose, maltotriose and sucrose were the substrates associated with these changes, which suggesting that *Saccharomyces* changed their metabolic pattern as the number of batches increased. We also observed that there was no significant difference in the flocculation level of London ale WLP013, while Czech Budejovice WLP802 started losing its flocculation levels after the fifth batch. In term of viability, London ale WLP013 strain dropped its viability in the final batch while Czech Budejovice WLP802 strain increased its viability after the first batch. No significant difference in alcohol by volume was observed in the two strains. The results of this study support our hypotheses that the metabolic potential and structural integrity of *Saccharomyces* strains were altered during serial repitching. In addition, YT plates represent a potential platform that can serve as an inexpensive tool to indicate a pitch number that is likely to produce undesirable beer for brewers.

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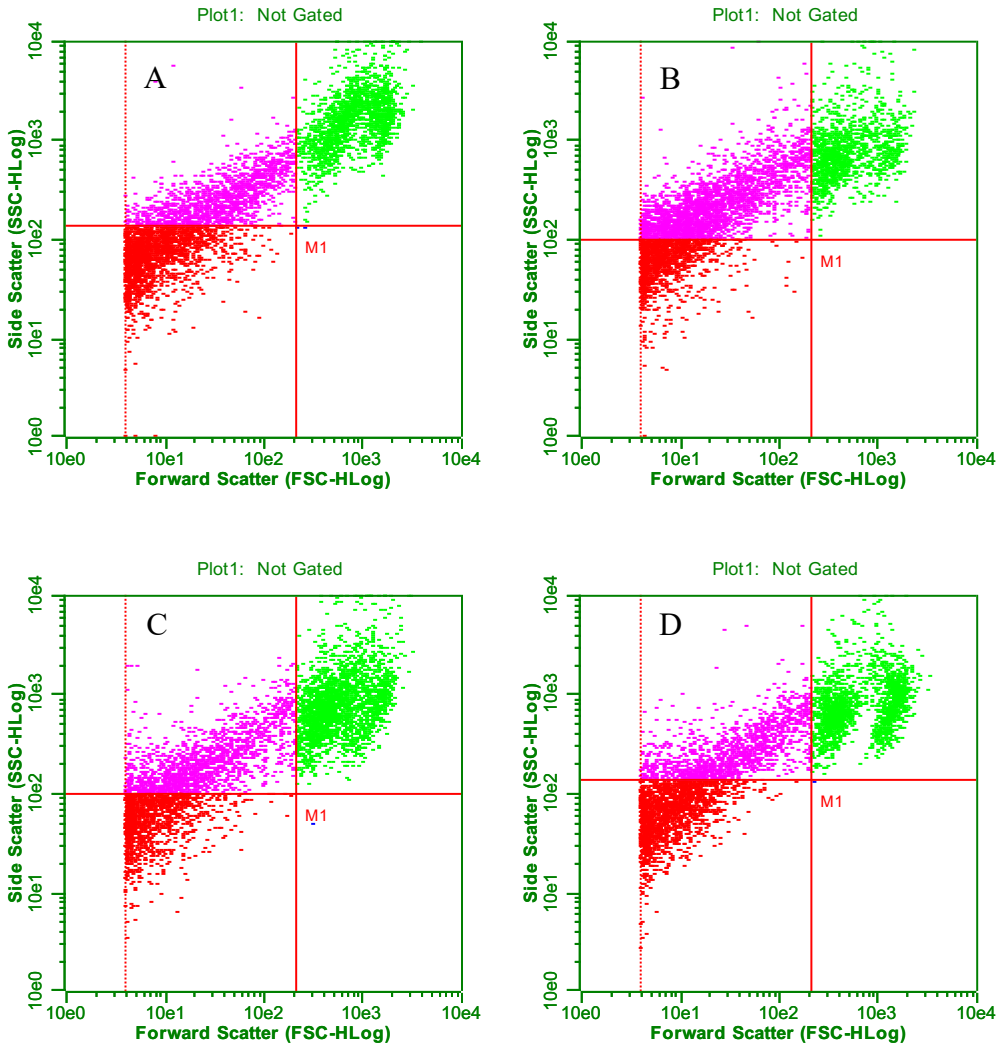
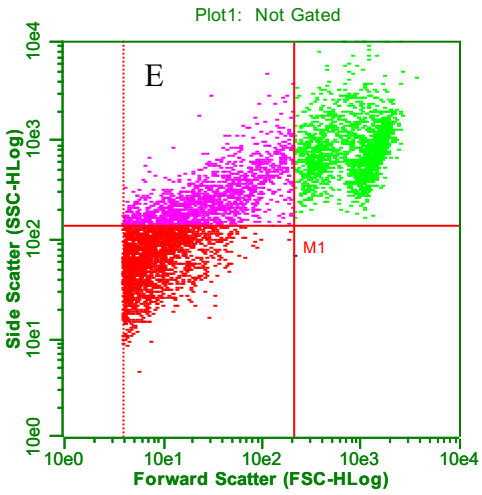
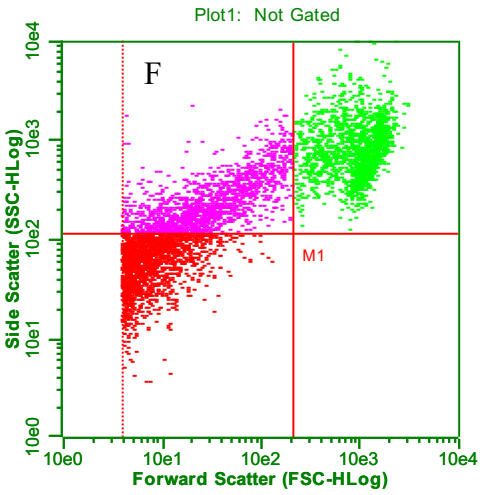
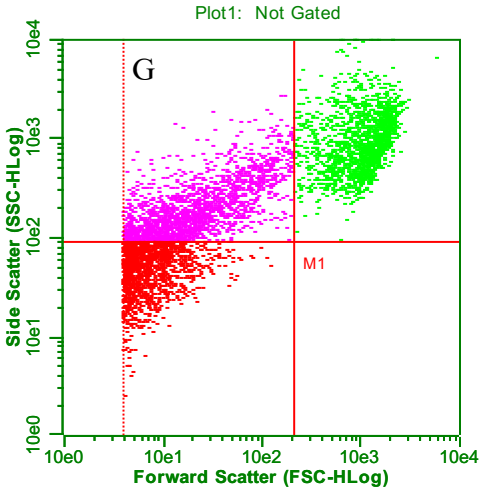
Appendix

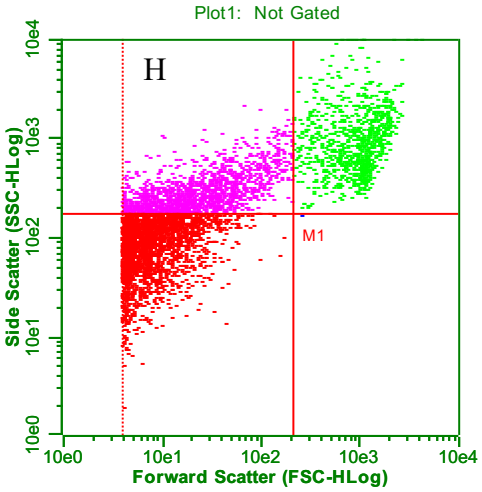
Appendix 1.1.1. Side scatter versus forward scatter for the first replication of Czech lager WLP802 strain throughout 8 batches of fermentation. A quad stat was applied to every sample, the upper and lower right are accounted as cell aggregates. Graphs A-H depict batches 1-8.



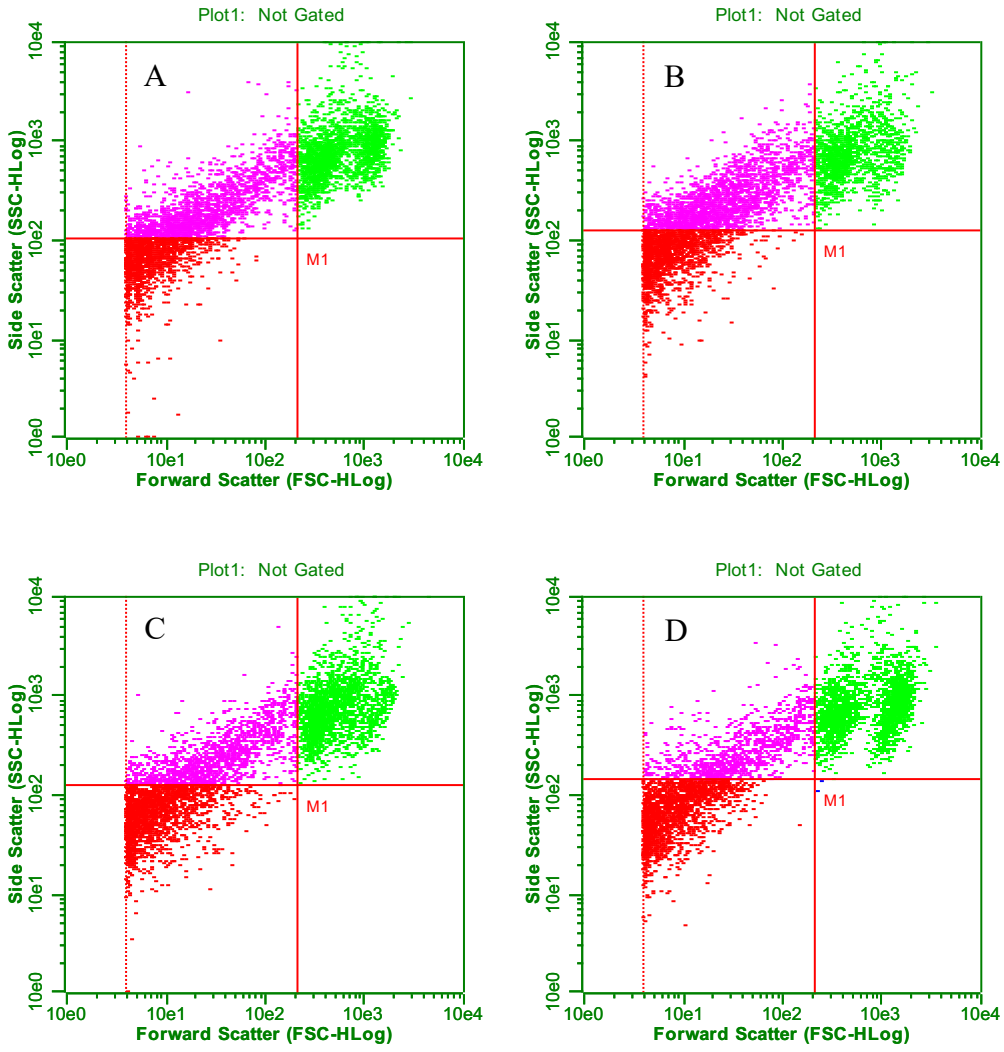

Plot1: Not Gated
E
M1
Side Scatter (SSC-HLog)
Forward Scatter (FSC-HLog)
10e0
10e1
10e2
10e3
10e4

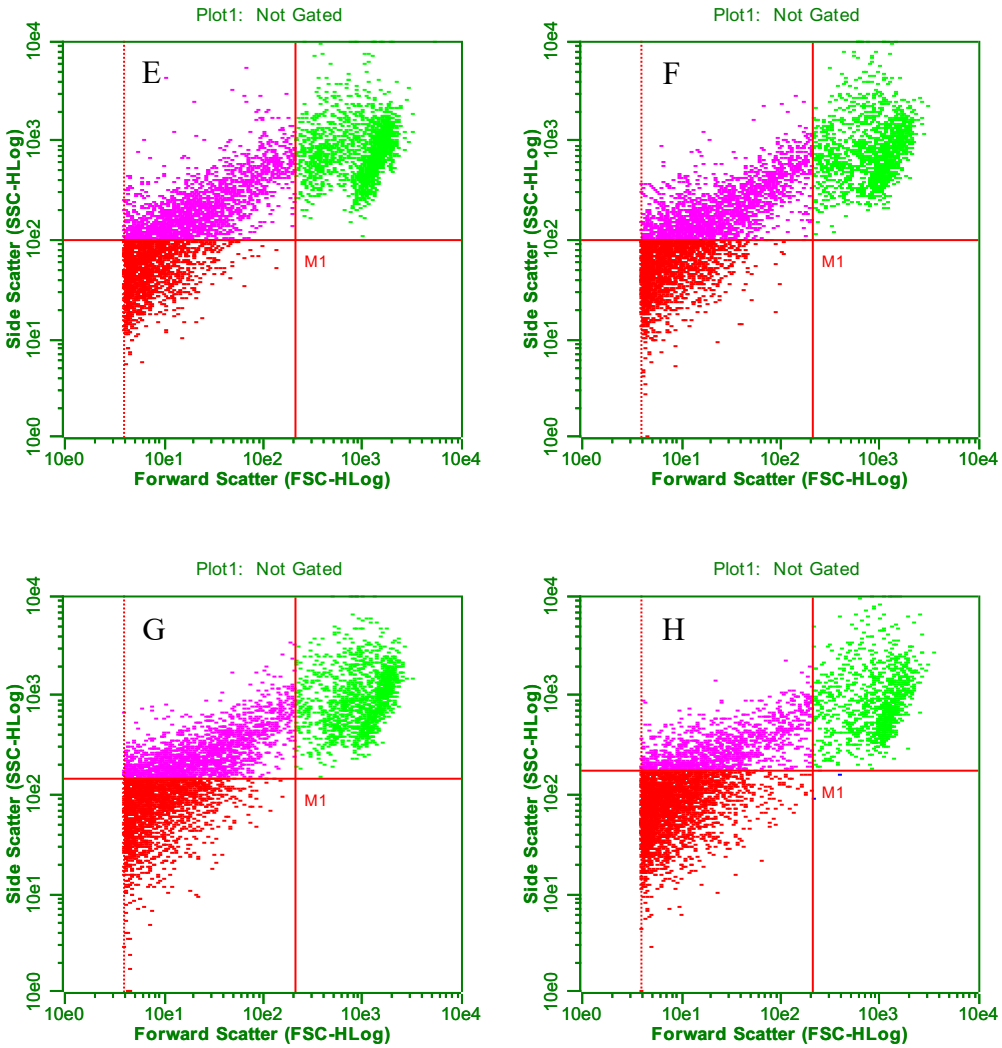

Plot1: Not Gated
F
M1
Side Scatter (SSC-HLog)
Forward Scatter (FSC-HLog)
10e0
10e1
10e2
10e3
10e4


Plot1: Not Gated
G
M1
Side Scatter (SSC-HLog)
Forward Scatter (FSC-HLog)
10e0
10e1
10e2
10e3
10e4


Plot1: Not Gated
H
M1
Side Scatter (SSC-HLog)
Forward Scatter (FSC-HLog)
10e0
10e1
10e2
10e3
10e4

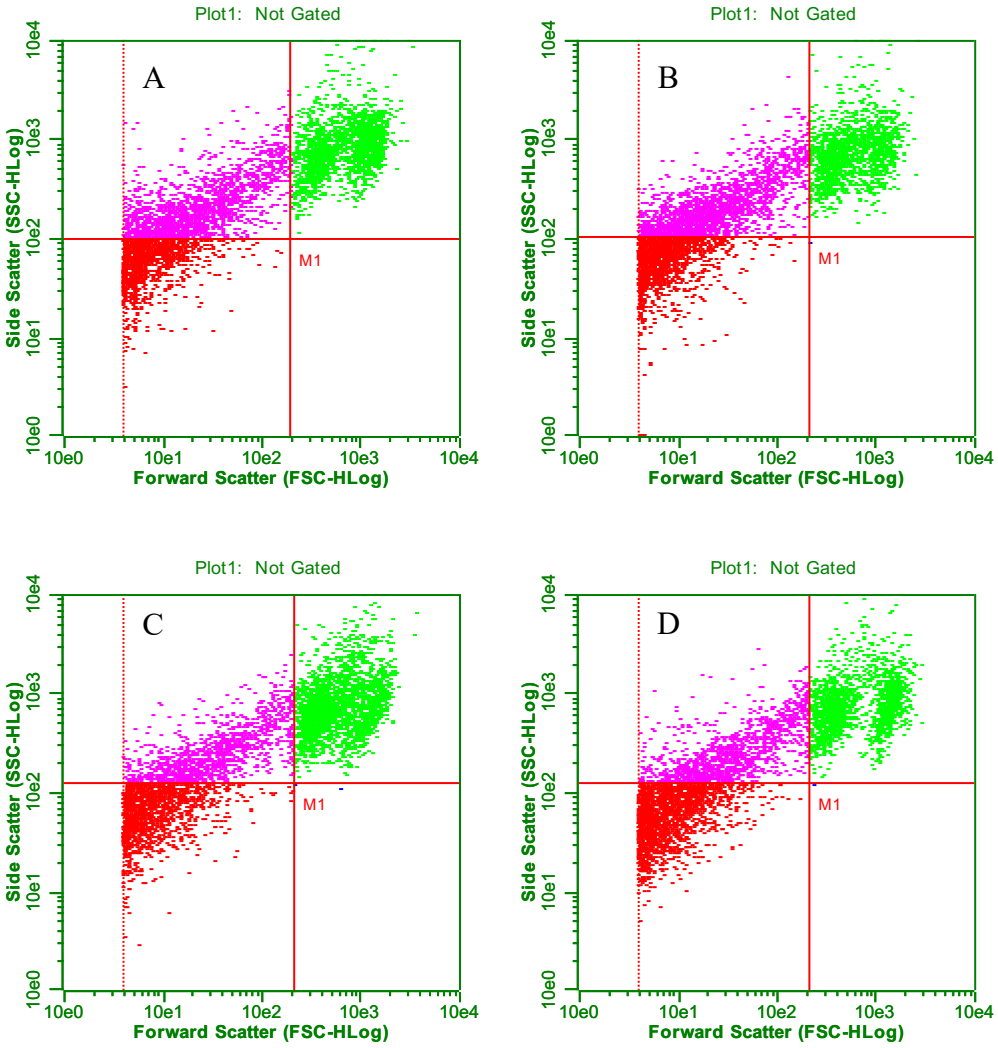
Appendix 1.1.2. Side scatter versus forward scatter for the second replication of Czech lager WLP802 strain throughout 8 batches of fermentation. A quad stat was applied to every sample, the upper and lower right are accounted as cell aggregates. Graphs A-H depict batches 1-8.

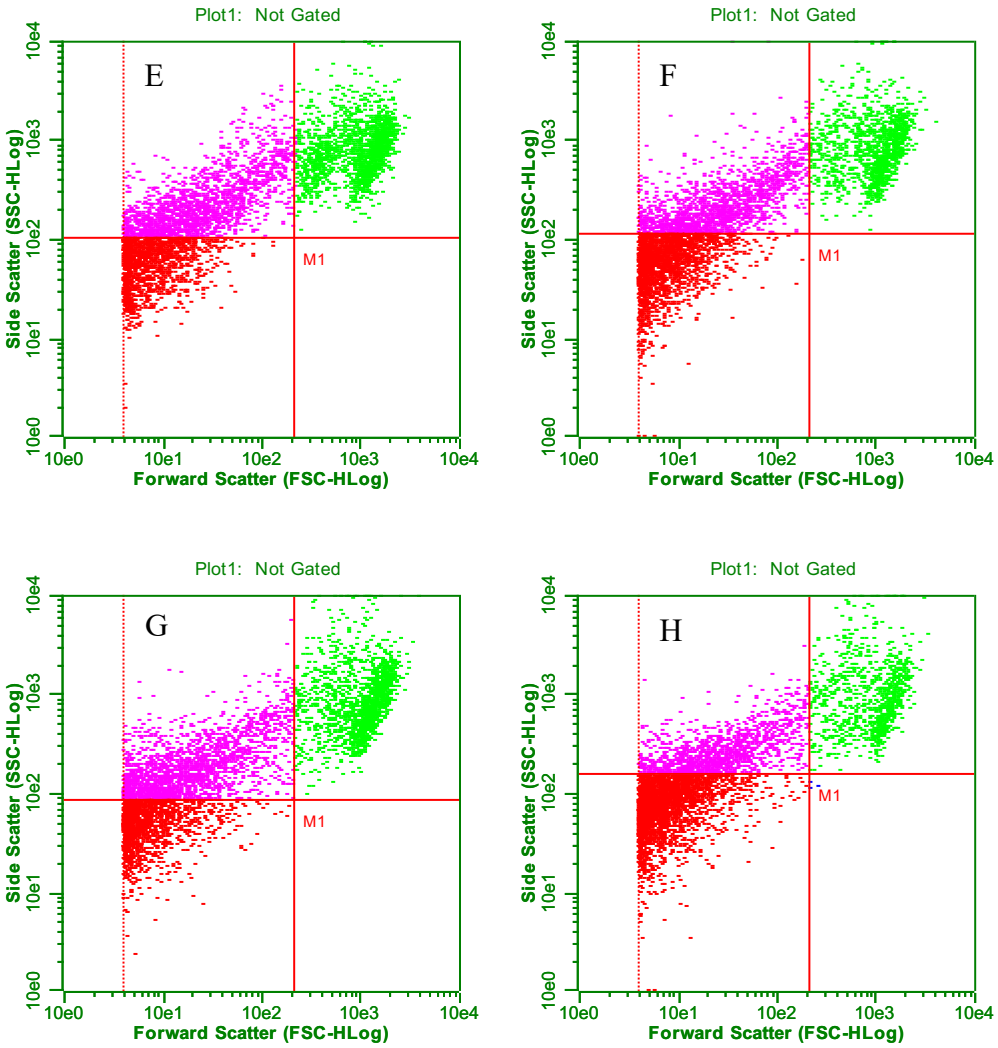


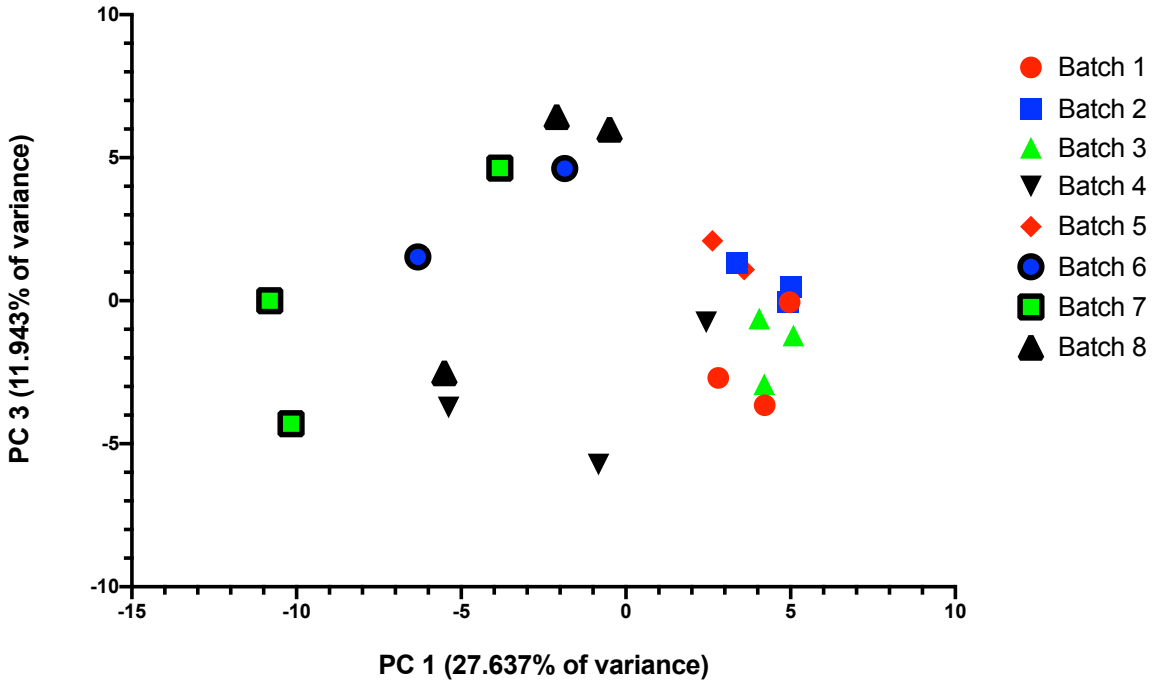


Appendix 1.1.3. Side scatter versus forward scatter for the third replication of Czech lager WLP802 strain throughout 8 batches of fermentation. A quad stat was applied to

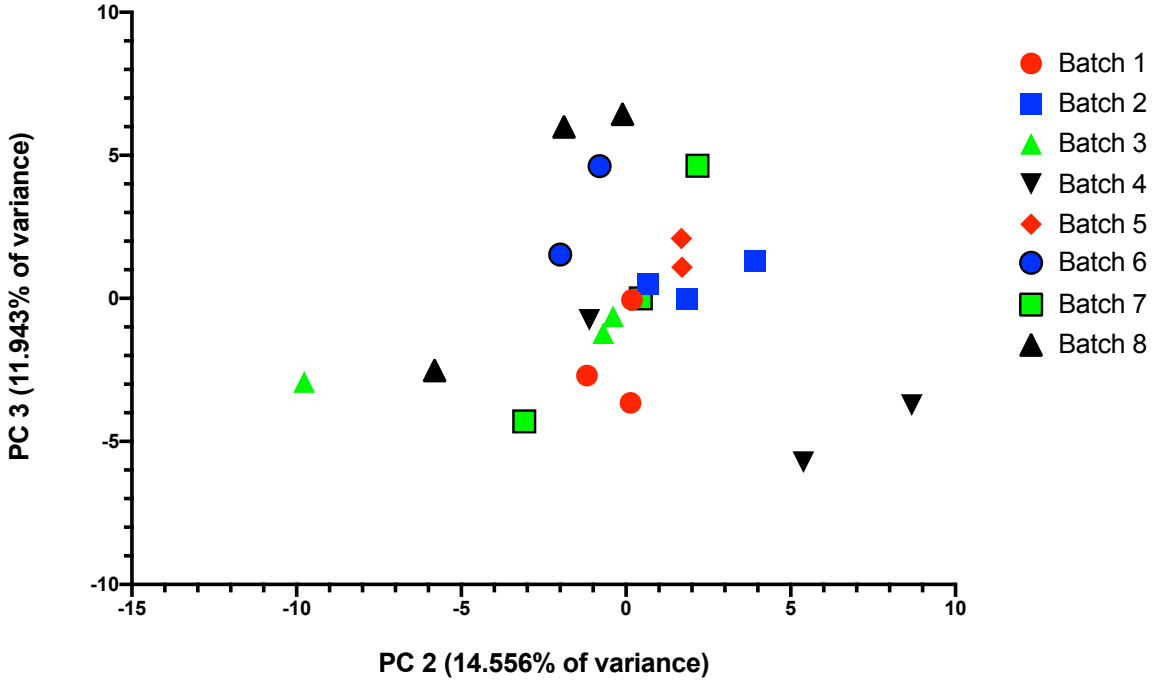
every sample, the upper and lower right are accounted as cell aggregates. Graphs A-H depict batches 1-8.



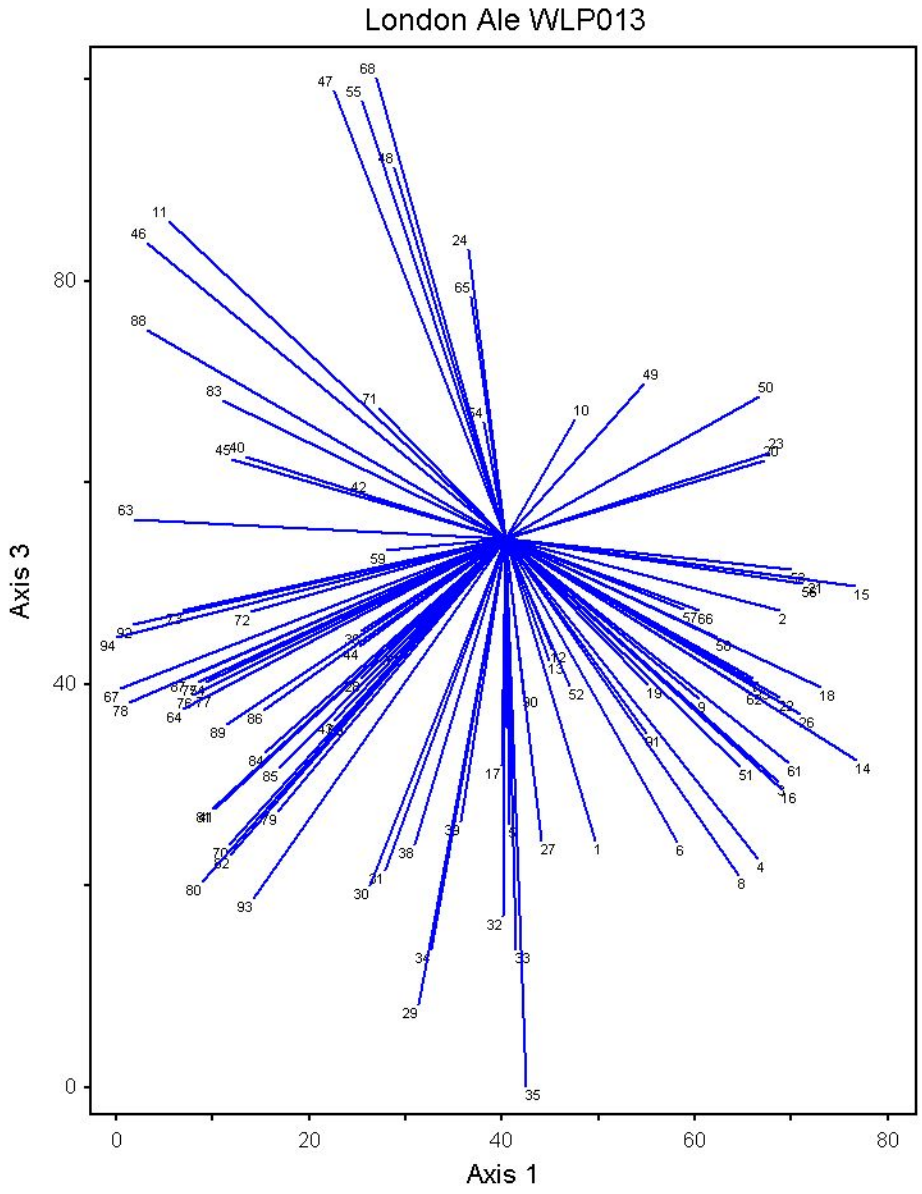

Plot1: Not Gated
E
M1
Side Scatter (SSC-HLog)
Forward Scatter (FSC-HLog)
10e0
10e1
10e2
10e3
10e4
Plot1: Not Gated
F
M1
Side Scatter (SSC-HLog)
Forward Scatter (FSC-HLog)
Plot1: Not Gated
G
M1
Side Scatter (SSC-HLog)
Forward Scatter (FSC-HLog)
Plot1: Not Gated
H
M1
Side Scatter (SSC-HLog)
Forward Scatter (FSC-HLog)



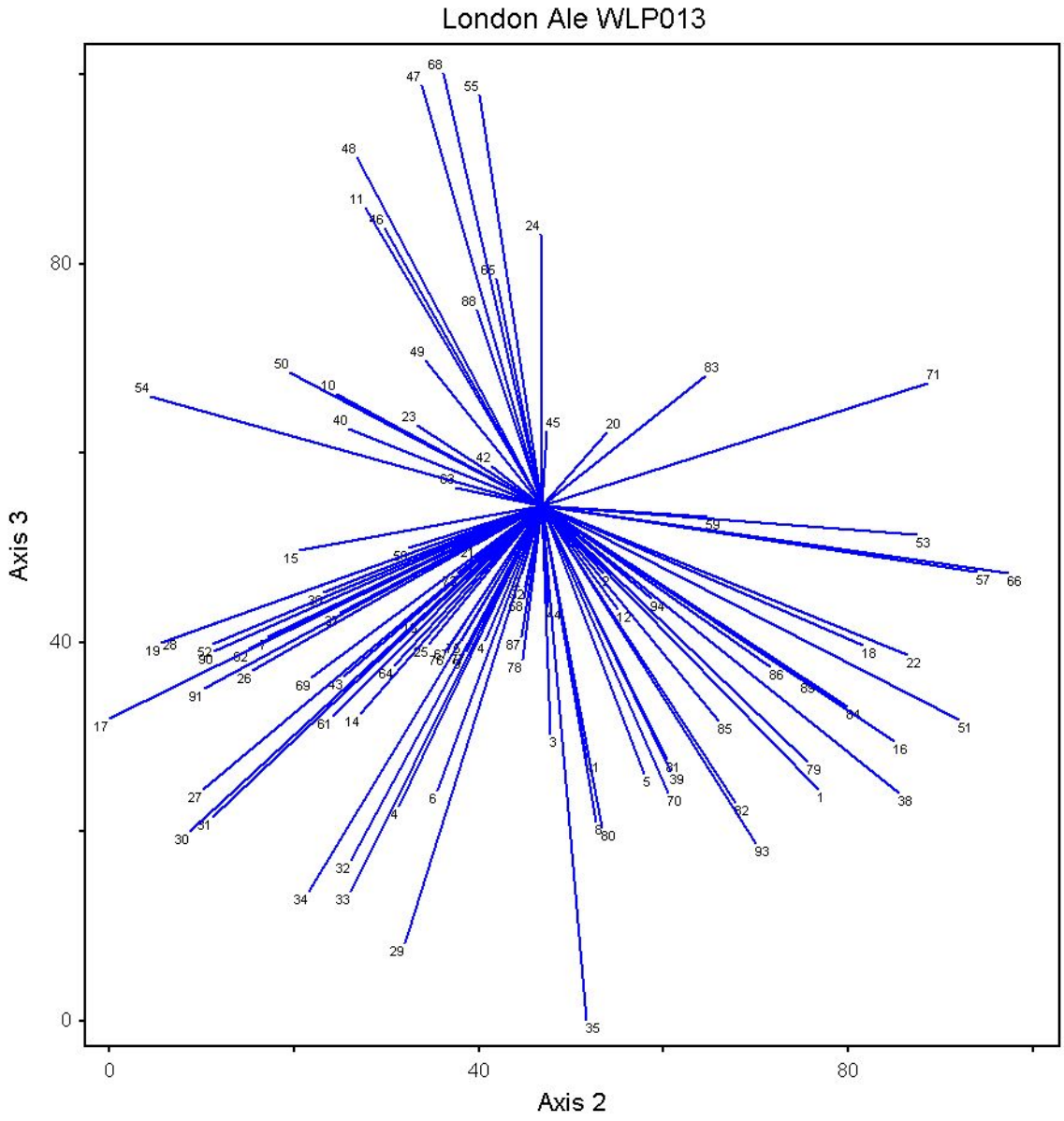
Appendix 1.2. Effects of serial repitching on metabolic potential of London ale WLP013 strain. Principle component analysis (principle component 1 (27.637% of variance) and principle component 3 (11.943% of variance)) of London ale WLP013 strain from 93 different biochemical tests. The eight different batches are indicated.



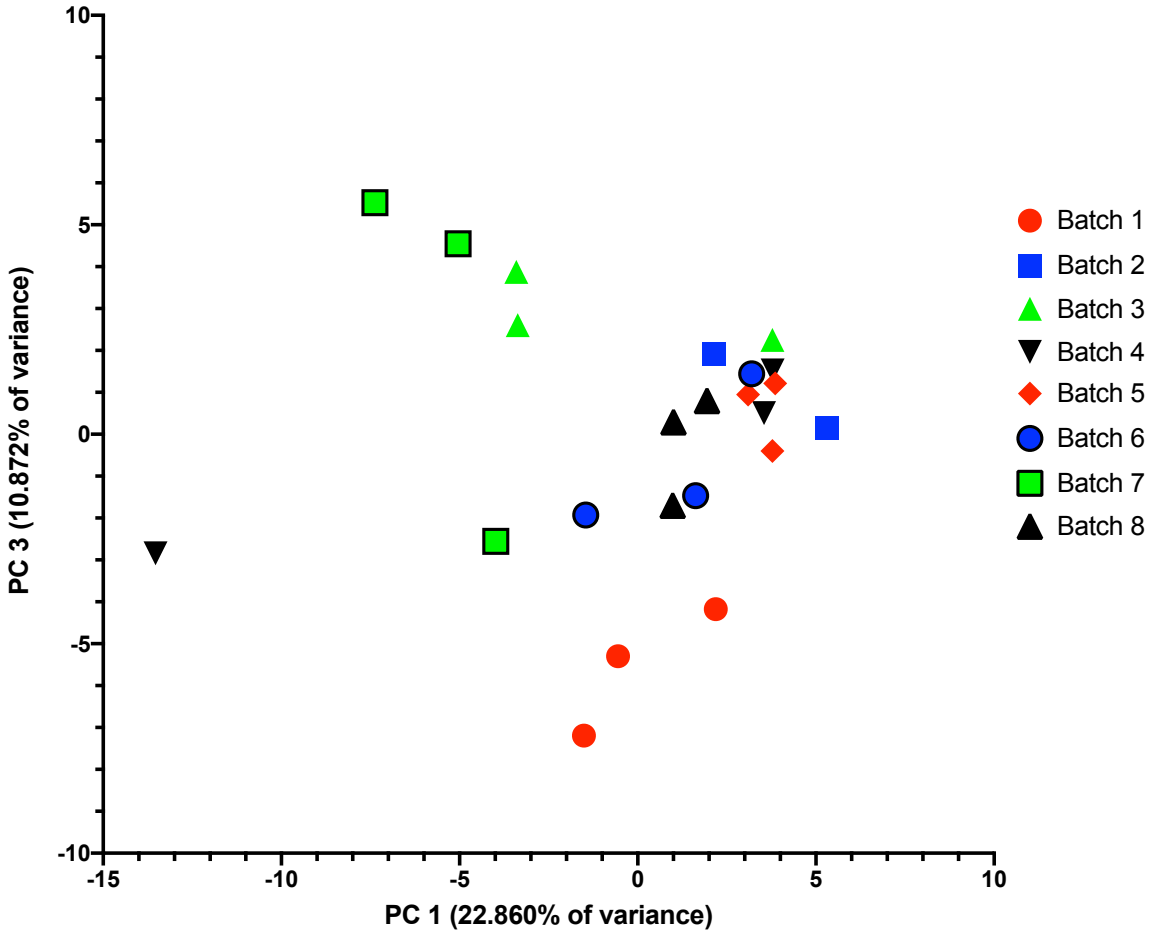
Appendix 1.3. Effects of serial repitching on metabolic potential of London ale WLP013 strain. Principle component analysis (principle component 2 (14.556% of variance) and principle component 3 (11.943% of variance)) of London ale WLP013 strain from 93 different biochemical tests. The eight different batches are indicated.



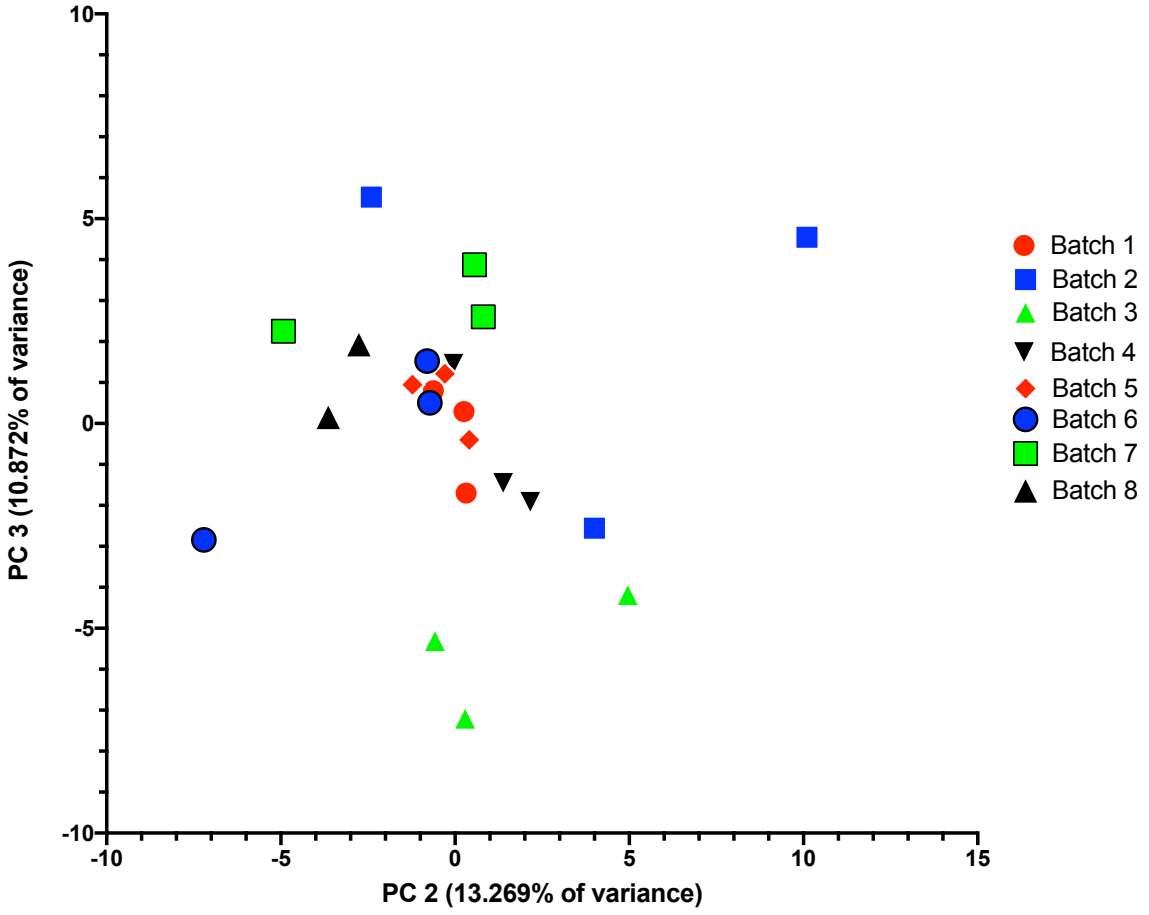
Appendix 1.4. Joint plot focusing on substrates distance of London Ale WLP013 strain between axis 1 and axis 3. Substrate dots correspond to the center of the samples. Distance between substrate and the inertia give an indication of the probability of substrate composition in samples. Maltose, maltotriose among other substrates were correlated to the differences between batches.



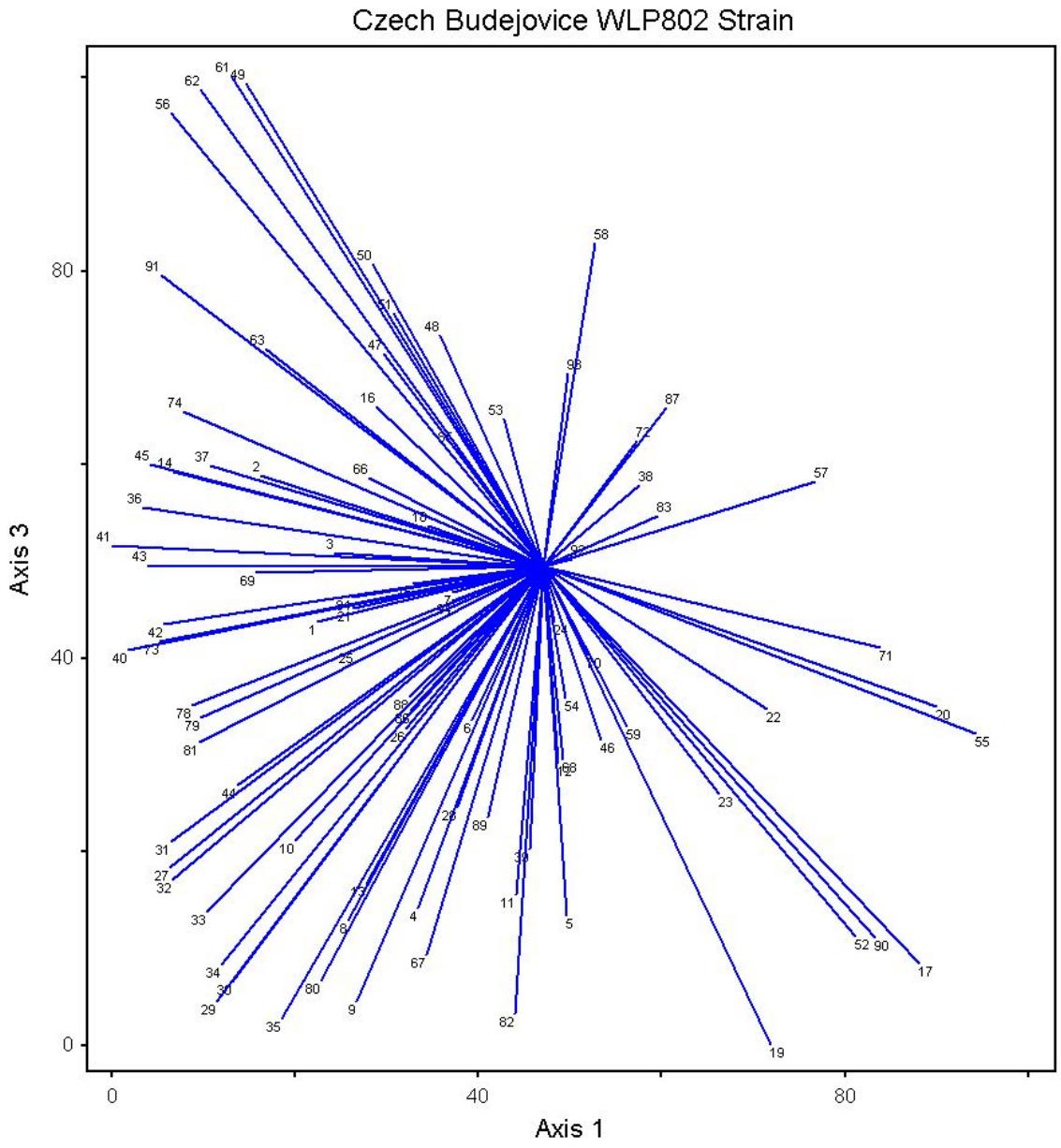
Appendix 1.5. Joint plot focusing on substrates distance of London Ale WLP013 strain between axis 2 and axis 3. Substrate dots correspond to the center of the samples. Distance between substrate and the inertia give an indication of the probability of substrate composition in samples. Maltose, maltotriose and sucrose were found correlated to the changes in metabolic potential among batches.



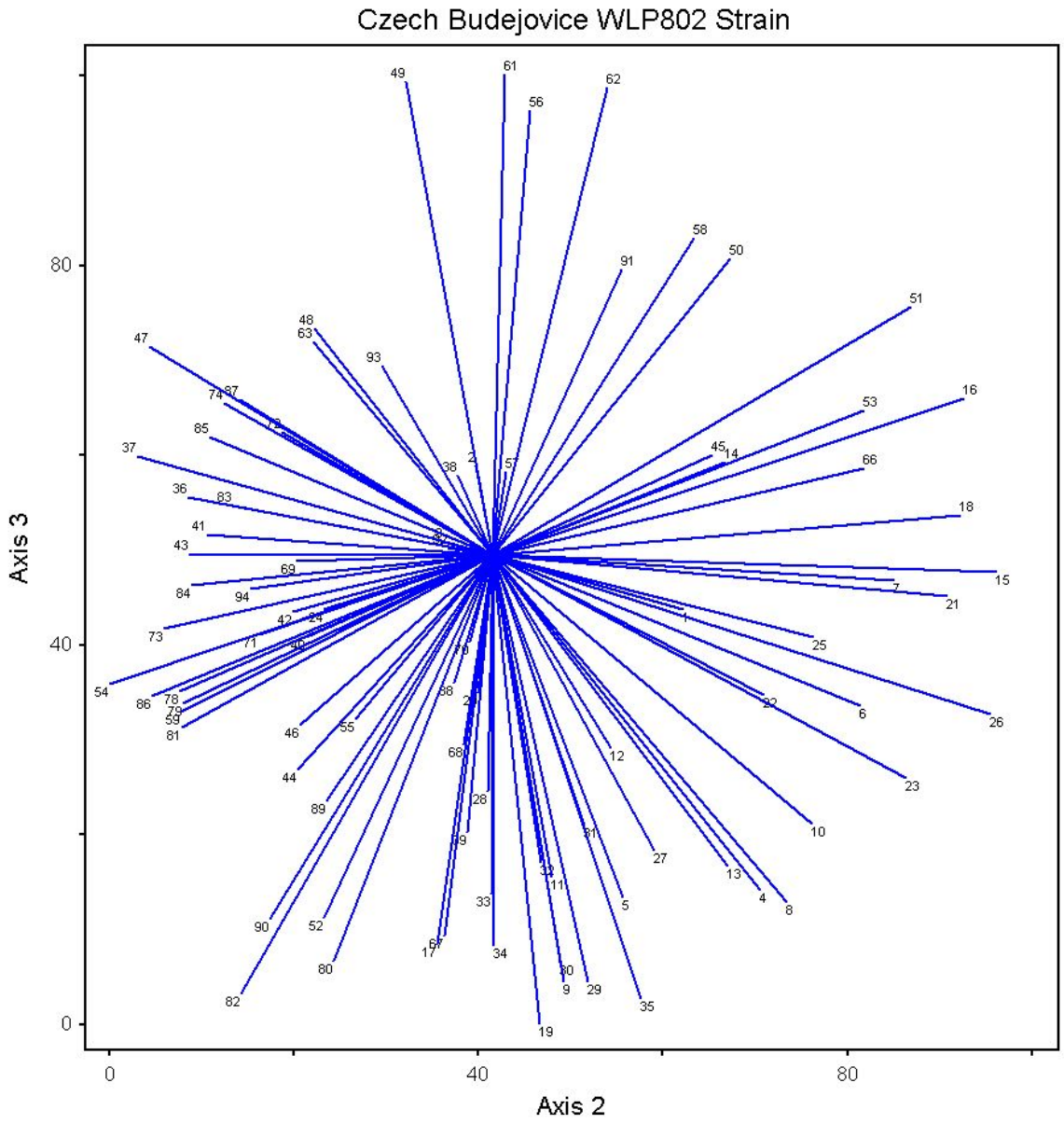
Appendix 1.6. Effects of serial repitching on metabolic potential of Czech Budejovice lager WLP802 strain. Principle component analysis (principle component 2 (22.860% of variance) and principle component 3 (10.872% of variance)) of Czech Budejovice lager WLP802 strain from 88 different biochemical tests. The eight different batches are indicated.



Appendix 1.7. Effects of serial repitching on metabolic potential of Czech Budejovice lager WLP802 strain. Principle component analysis (principle component 2 (13.269% of variance) and principle component 3 (10.872% of variance)) of Czech Budejovice lager WLP802 strain from 88 different biochemical tests. The eight different batches are indicated.



Appendix 1.8. Joint plot focusing on substrates distance of Czech Budejovice lager WLP802 strain between axis 1 and axis 3. Substrate dots correspond to the center of the samples. The length of the line represents Euclidean distance value and indicates the degree to which that substrate influences the principle component analysis. The vector also indicates the substrate respected with principle components.



Appendix 1.9. Joint plot focusing on substrates distance of Czech Budejovice lager WLP802 strain between axis 2 and axis 3. Substrate dots correspond to the center of the samples. The length of the line represents Euclidean distance value and indicates the degree to which that substrate influences the principle component analysis. The vector also indicates the substrate respected with principle components.