Master's Thesis in Applied Microbiology

Method development of flow cytometry analysis of yeast and lactic acid bacteria viability for alcohol fermentation

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Abstract

The usage of yeast in conventional brewing techniques involving alcoholic fermentation is an age-old process. Analyzing the viability and obtaining physiological information regarding the microbial diversity of the fermentation samples plays a pivotal role in obtaining real-time information as well as developing a more efficient process design. While traditionally, methods like methylene blue cell staining and/or colony counting techniques provide viability information, their arduous nature and general inconvenience has resulted in the adoption of flow cytometry to not only analyze individual cells but also in-depth cell physiological information in a matter of minutes. The primary focus of this project was to develop a method which utilized flow cytometry to monitor the microbial diversity of the complicated fermentation matrix, in particular the cell concentration and viability of the yeast *Saccharomyces cerevisiae* and *Lacticaseibacillus casei.* The developed method offers the quantitative viability analysis of rehydrated freeze-dried yeast and potential lactic acid bacteria present in the fermentation matrix through flow cytometry and better understand the microbial interactions within the fermentation tanks.

Keywords: Flow cytometry, yeast, lactic acid bacteria, fermentation, cell viability

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List of abbreviations

1. ADY – Active dry yeast
2. FC – Flow cytometer
3. FCM – Flow cytometry
4. FDA – Fluorescein diacetate
5. FL1 – Fluorescence 1 (green)
6. FL3 – Fluorescence 3 (red)
7. FSC – Forward scatter
8. LAB – Lactic Acid Bacteria
9. PI – Propidium iodide
10. SSC – Side scatter
11. TAC – The Absolut Company
12. TO – Thiazole orange

1. Introduction

1.1 Project background

The Absolut Company is a world-renowned producer of vodka that today holds the third place when it comes to the biggest spirit producers in the world. While creativity and innovation are the keys to growth, ensuring every step of the production process is optimized to be the most efficient version of itself is paramount to ensuring energy efficiency and thereby sustainability. The fermentation phase is one of the crucial steps in the production process of ethanol and by monitoring the viability level of the yeast, invaluable information can be provided regarding the ongoing process and can also be a valuable control parameter when performing future fermentation optimizations. On the other side, the viability of lactic acid bacteria is also important in the fermentation process by acting as an indicator of arising hygienic problems that need to be looked after by additional- or more thorough cleaning steps.

While cultivation-based techniques to analyze microbial numbers such as determining the number of colony forming units provide important information, there are several drawbacks. For instance, the long waiting times for colony growth, the time, energy, and resource requirement etc., all point to the room for alternative methods for process optimization such as, for example flow cytometry. This master's thesis is a collaboration between The Absolut Company and Lund University, which aims to develop a fully optimized viability analysis technique utilizing flow cytometry that could be used to analyze fermentation samples at ease, in the future.

1.2 Aim

This project aims to develop a flow cytometry analytical method to quantitatively identify yeast and Lactic Acid Bacteria simultaneously from a complicated fermentation "mash" matrix. Fermentation mash consists of wheat flour, water, and a combination of enzymes during the fermentation of ethanol. Microbial analysis is needed to monitor the changes in the microbial population in real-time, as well as to simultaneously carry out the viability analysis of the detected cell populations. My project aimed furthermore to compare the results obtained from flow cytometry analysis to traditional colony forming unit method as well as hemocytometer microscopic analysis.

In order to achieve this goal, the initial phase of the project was focused on understanding the working of the flow cytometer and determining the suitable dilution which could be analyzed using the flow cytometer. Later, the viability level of the yeast cells was quantified. This process was repeated for lactic acid bacteria and samples containing a mixture of yeast and lactic acid bacteria. The obtained results were analyzed and studied to optimize the instrument's settings and create a template which could later be used to analyze fermentation samples quickly and efficiently in the future. The results obtained, along with background theory detailing the various mechanisms and microorganisms involved have been compiled into the following thesis report.

2. Background theory

2.1 Fermentation

The process of fermentation has been utilized by mankind for centuries despite a lack of clear understanding of the mechanisms involved in the actual process. But due to the incessant advance in the science of applied and fermentation microbiology we now have a clear understanding of the microorganisms involved as well as the plethora of ways through which fermentation can be utilized (Mansi et al., 2018). While fermentation started off as a foolproof way to preserve a number of foods, it gradually branched into alcoholic fermentations involved in breweries and winemaking (Ross, Morgan and Hill, 2002). The expansion in the usage of various fermentation techniques created a need to intensively study the growth and metabolism of yeast, *Saccharomyces cerevisiae*, which has now led it to be one of the most thoroughly studied of all eukaryotic cells (Boulton and Quain, 2008). Despite all the existing knowledge regarding the culture, growth, and metabolism of yeast, several analytical techniques remain relatively under-researched and will be the focus of this thesis report.

2.2 Analysis of fermentation

In order to make informed decisions in regard to production, a thorough analysis including both chemical and microbial analyses of fermentation is necessary. The microbial population keeps varying throughout the course of fermentation and therefore poses multiple challenges when a certain population needs to be quantified (Rodriguez and Thornton, 2007). Traditionally, the estimation of colony forming units (CFUs) and other culture-based analytical methods provides an insight to the estimation of viable cells, but these methods are not only subject to long incubation periods (Deere et al., 1998) but also only provide an estimate of the number of live cells. Providing no information regarding the dead cells. Furthermore, the presence of the desired cells in a complicated environment poses a certain limitation in the microbial analysis i.e., the yeast and lactic acid bacteria are present in a complicated matrix known as fermentation mash comprising of water, wheat flour, and a mixture of enzymes such as alpha-amylase, gluco-amylase and protease. Isolating the cells from the background mash debris requires meticulous sample preparation before analysis. Despite the careful sample preparation, the possibility that the bacteria may be too few to count or may just be too small to identify under the microscope (Rodriguez and Thornton, 2007), poses yet another hindrance in the prospect of simultaneously quantifying and analyzing the desired microorganisms via microscopy. These reasons point to flow cytometry as a prospective candidate due to its rapid analytical time, estimation of live and dead cells, non-requirement for growth assays or long incubation periods as needed for colony counting techniques, and also the unbiased analysis of live bacteria that cannot be cultured (Khan, Pyle, and Camper, 2010).

2.3 Lactic Acid Bacteria in fermentation

Lactic Acid Bacteria (LAB) are not uncommon in alcoholic fermentations due to their ability to thrive in environments of low pH (between 6 to 3.5) and lack of oxygen requirement. Although LAB can have beneficial effects in alcoholic fermentation i.e., the malolactic fermentation of wine (Priest, 2005), their effects can generally be viewed as undesirable due to their ability to compete with the yeast cells, thereby

varying fermentation times and reducing the overall alcoholic yield. The potential utilization of LAB in fermentation would require detailed information regarding the starter culture used as it affects the flavor profile of the end product (Overton, 2015). In The Absolut Company (TAC), the presence of LAB is characterized by an increase in organic acids in the fermentation samples, when analyzed via HPLC (High Performance Liquid Chromatography) where a single run takes approximately 50 mins.

2.4 Flow cytometry

Flow cytometry involves creating a liquid suspension of the desired sample. This liquid sample is then moved upwards via a capillary tube in the flow cytometer within a liquid stream known as sheath fluid. As the cells move in a single file, cell by cell, they are interrogated with the help of a laser. Upon contact with the cell, the light emitted by the laser is now also in contact with the fluorochrome which now has the energy to get excited and also emit light. This light gets scattered in two predominant directions, commonly referred to as Forward angle light scatter (Forward Scatter or FSC) or Side angle light scatter (Side Scatter or SSC). These two parameters aid in differentiating between various cell populations and obtaining a rough idea about the size and granularity of each population that has been analyzed. This can be considered the first step in estimating the viability of desired cells, i.e., isolating the desired cells from a complicated matrix followed by identifying its concentration. The "desired cells" in this report will initially focus on Active Dry Yeast, then followed by Lactic Acid Bacteria. The fact that yeasts are generally much larger than LAB is taken advantage of for FCM analysis. Yeasts are typically around 3 – 4 µm (Yeasts, n.d.), while LAB tend to be around 0.7 to 1.1 µm (*Lactobacillus Casei ATCC 334*, n.d.)

Once the cells have been isolated from the fermentation mash matrix and separated by size and granularity, the final and more specific parameter used by the flow cytometer can be referred to with an umbrella term called fluorescence. Cells that have been stained with specific viability dyes emit fluorescence signals at different wavelengths as they pass by the laser. As the cells move in a single file, valuable information from each individual cell can be collected in a short span of time. Information such as individual cell physiology, cell membrane integrity etc. can be obtained at rapid speeds (Egli and Kotzsch, 2015).

2.5 Advantages of Flow Cytometry

While traditionally, colony counting methods are utilized to acquire data of this nature, flow cytometry (FCM) poses significant advantages over most existing methods for determining cell concentrations and information regarding various cell physiology parameters. The single most attractive feature of flow cytometry is the speed at which a vast amount of information can be acquired. Not only can all the above-mentioned data can be obtained within a matter of minutes, but the volume of sample also required is minimal (starting at less than 1 µL, varying with cell concentration in the sample and type of flow cytometer). Moreover, multiple strains can be analyzed during the same short span of time while providing precise and detailed information about each desired cell population which is made possible due to the availability of several staining dyes. While a flow cytometer can initially pose a significant capital requirement, it can be considered an investment as the amount of time, labor, energy, resources, and space saved over a period of time outnumbers the benefits of conventional plating and colony counting techniques. Moreover, in the case of viability assessment of cells, no information regarding the "dead"

cells can be obtained. Additionally, the ability to visualize and obtain information regarding two different cell parameters at once adds to the list of FCM advantages (Lugli, Roederer and Cossarizza, 2010)

FCM analysis also provides a quantitative analysis of both yeast and Lactic Acid Bacteria which cannot be achieved by conventional plating techniques. While light and other fluorescence microscopic techniques can be used to achieve quantitative cellular data as well, the cell count for which FCM provides data vastly outnumbers the cells that can be manually counted via microscopic techniques (Georgieva et al, 2008*)*, with the obvious advantage of time and almost no manual labor requirement, in comparison. Moreover, since each individual cell is being analyzed by the flow cytometer, it greatly reduces the potential background fluorescence from unincorporated dyes present in the solution (Overton, 2015) or in this case, the mash matrix.

Moreover, bacterial populations may not only be too small but also too few to count via microscopic methods as they are present at around a 100-fold lower concentration than yeast. The existence of Viable but nonculturable (VNBC) bacteria is yet another drawback in relying on standard colony counting techniques for an accurate count of the desired microorganism (Rodriguez and Thornton, 2008).

2.6 Fluorescent stains provide detailed information.

As mentioned earlier, complex cellular characteristics of every cell that has been analyzed by FCM can also be obtained by utilizing a plethora of fluorescent dyes that are now available. These dyes help assess the viability of the desired cells by indicating whether they are “alive”, “dead”, damaged, or quiescent. Some of the commonly used “live” cells staining viability marker dyes include SYBR green, Thiazole orange (TO), Carboxy Fluorescein Diacetate (CFDA), Fluorescein Diacetate (FDA) etc. Thiazole orange is a viability marker which works by emitting a significantly higher fluorescence upon interaction via intercalation with nucleic acids (see figure 1) such as double stranded DNA (Deoxy Ribonucleic Acid) and RNA (Ribonucleic Acid) (Suss, Motiei, and Margulies, 2021). Derivatives of fluorescein are well-known cell viability markers. This is a direct consequence of their esterase activity wherein the dye is immediately taken in by the cell after which the cellular esterases cleave the compound, thereby releasing the fluorescein which is membrane impermeable. Moreover, the exclusion of PI alone can be used as a criterion to assess the viability level of a cell (Overton, 2015).

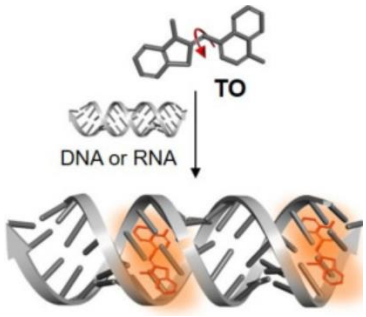

Figure 1: Thiazole orange intercalating or stacking in between the grooves of nucleic acids (Suss, Motiei, and Margulies, 2021)

Arguably, the most commonly used counterstain for fluorescein derivatives is Propidium Iodide (PI) which is able to successfully stain intercalate with the DNA of certain cells whose cellular membranes have been compromised or damaged, allowing the dye to penetrate into the nucleoplasm, thereby staining the DNA. (Oliveira, Crespo, and Pereira, 2019).

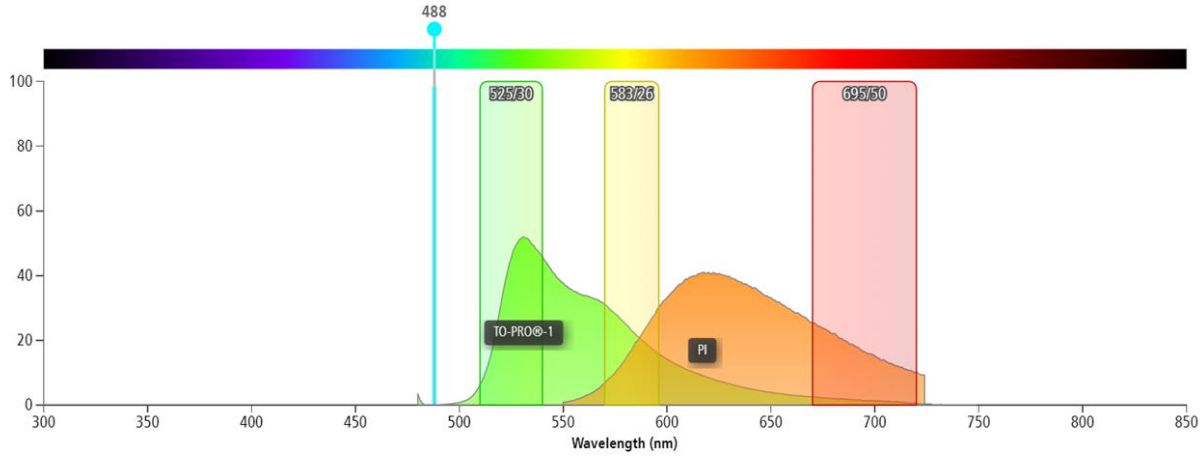

Figure 2: Emission wavelength range for viability markers thiazole orange (TO) and PI respectively, along with the three filters present in the Guava EasyCyte SL flow cytometer in a 488 nm blue laser line (*BD Spectrum Viewer*, n.d.)

Understanding the absorbance and emission characteristics of a fluorescent dye is pivotal to ensure that the results obtained are completely accurate. The range between which the dye emits fluorescence, and the exact wavelength at which it peaks function as an accurate guide to selecting dye suitable for the flow cytometer at hand. For instance, the dye PI peaks at 617 nm emission maximum (Vandewalle, n.d.) (see figure 2), so the closer the filter in the flow cytometer is to this specific wavelength, the stronger PI's emission fluorescence will be.

A dual staining system consisting of both live, dead, and thereby damaged cells staining dyes are specifically attractive as they can aid in obtaining a clear separation between the live and dead populations, while also providing information about the damaged cells. However, it is important to note that the flow cytometer used has filters that are optimized for two stains in question. If not, it might lead to the emission fluorescence from one dye out shadowing the other. The dual staining system in the case of both the yeast and bacterial cells involves a stain which acts as a marker of viability and a marker of damaged membrane integrity i.e., cells with compromised membranes, indicating "dead" cells.

Cell viability is the only characteristic of interest for this report therefore the dyes used for staining are a mixture of a stain and a counterstain to indicate the membrane intact and metabolically active ("live"), membrane compromised ("dead") and membrane damaged cells of the yeast and bacterial cells.

3. Materials and methods

3.1 Development of Flow Cytometry (FCM) analysis technique

3.1.1 Flow cytometry analysis

3.1.1.1 Set-up of Guava EasyCyte™

A *Guava EasyCyte™ SL* flow cytometer *(Luminex Corporation, Technology Boulevard, Austin, Texas, U.S.A.)* equipped with a blue 50 mW laser line with sample concentration analyses limits ranging from $1X10^4$ to $5X10^5$, for the most accurate analyses results. The flow cytometer was also equipped with the following emission filters:

Instrument: Guava EasyCyte SL flow cytometer			
Laser line	488 nm		
Emission filters	525/30 (FL1)	583/26 (FL2)	695/50 (FL3)
Fluorochrome	FDA, Thiazole orange		PI

Table 1: Information regarding flow cytometer specifications.

3.1.1.2 Instrument calibration

Prior to analyzing the samples, the equipment was calibrated using the *Guava® easyCheck™ Kit (4500-0025)* provided by *Luminex Corp.* In order to ensure the optimum performance and thereby obtain accurate results regarding the scatter and fluorescence information from the flow cytometer. This was carried out by combining 380 µL of *Guava® Check Diluent* of *LOT number B95251* with 20 µL *Guava® easyCheck™ Beads* of *LOT number B93857*.

In order to efficiently quantify and analyze the yeast and bacterial populations, the equipment was optimized via the creation of templates for yeast cells separately and samples containing both yeast and bacterial cells.

3.1.2 Sample preparation

3.1.2.1 Active Dry Yeast (ADY)

The Active Dry Yeast (ADY) strain *Saccharomyces cerevisiae* has been used throughout the experimentation. 0.5 g of ADY was mixed with 4.5 mL of tap water treated by TAC water treatment plant in a test tube in order to create the first dilution (1:10). In order to effectively rehydrate the freeze-dried active dry yeast, the test tube was submerged in a 34°C water bath *(Grant - T100)* for 15 mins, gently vortexing the sample every 5 mins using a *Heidolph REAX 2000 (No. 541-19000-00).* This sample was

serially diluted up to 10^{-5} dilutions by transferring 0.5 mL of the previous dilution to a test tube containing 4.5 mL of tap water, with gentle vortexing in between each transfer of which is the required dilution for the flow cytometer (FC) used as it contains the optimum concentration of cells that can be analyzed comfortable within the event count of the flow cytometer. A *Guava EasyCyte™* flow cytometer (Luminex Corporation, Technology Boulevard, Austin, Texas, U.S.A.) was used to analyze the samples and the *FlowJo version 10.9.0* software was used for data analysis. To execute the viability analysis of ADY present in fermentation mash, the ADY was initially rehydrated per the above-mentioned protocol. This sample of 1:10 diluted yeast and water was gently vortexed before 0.5 g of the rehydrated yeast was transferred to a test tube containing 4.5 g of mash sample. This yeast-mash mixture was once again vortexed before 0.4 mL of the sample was transferred to a test tube containing 4.5 mL water. This sample was then filtered using a 20 μm filter provided by *Sysmex CellTrics® of LOT number CT221129* in order to separate the desired cells, in this case the rehydrated active dry yeast cells from the mash matrix. This sample was further diluted up to 10^{-5} dilution and 2000 events per run were analyzed at a low flow rate.

3.1.2.2 Lactic Acid Bacteria (LAB)

To create a Lactic Acid Bacteria sample that can be analyzed by the flow cytometer, a stock solution of a commercial cultured yoghurt product containing a concentration of 10^{10} *Lacticaseibacillus casei* cells was used. The tap water used to serially dilute the bacterial sample was initially filtered using a 0.2 μm *IC MILLEX®-LG filter* of *LOT number R9HA53233* in order to effectively filter out pre-existing bacterial cells in the water. The sample was serially diluted to a dilution of 10^{-4} as this was previously estimated to be the optimum concentration for both the flow cytometer due to the cell concentration in the product as well as for method development. After obtaining the 10^{-2} dilution, the sample was filtered with a 20 μm filter and diluted further until the desired cell concentration was acquired. The bacterial sample was also individually tested with the fermentation mash sample before being analyzed with both the rehydrated yeast cells and mash. 0.5 mL of bacterial sample from commercial cultured yogurt product was diluted in 4.5 mL of fermentation mash and vortexed well to ensure the sample is homogeneously mixed. 0.5 mL of this sample was further diluted in 4.5 mL of tap water that had been previously filtered with a 0.2 μm filter. This sample was further serially diluted to a 10^{-4} prior to FC analysis and 5000 events per run were analyzed at a low flow rate.

3.1.2.3 Active Dry Yeast and Lactic Acid Bacteria

Finally, in order to develop a FCM analysis method to simultaneously and quantitatively analyze both the yeast and bacterial cells present in the mash samples obtained from the fermentation tanks of The Absolut Company, a laboratory scale replica of the mash was created. Initially this method was tested excluding the fermentation mash, analyzing samples containing ADY and LAB serially diluted in TAC treated tap water. Later, fermentation mash was included in the viability analysis, and this was done by combining 0.5 g of ADY with 4.5 mL of treated tap water and rehydrating the freeze-dried yeast cells in a 34°C water bath for 15 mins, and gently vortexed every 5 mins. Post rehydration, 0.5 mL of the diluted rehydrated yeast was combined with 4.0 mL of fermentation mash and 0.5 mL bacterial cell culture. This mixture was vortexed until a homogenous sample was obtained and serially diluted until the desired dilution of 10^{-5} for yeast, and 10^{-4} for LAB was obtained. This sample was later stained and analyzed using the flow cytometer at 5000 events per run, at a low flow rate.

3.1.2.4 Controls set-up

In order to successfully establish positive and negative yeast cell controls for the flow cytometry method development, the following steps were undertaken. 2.5 mL of the 10^{-5} dilution was pipetted out into a separate test tube. A small beaker of water was heat treated and removed from the heat once it began visibly boiling. The test tube containing the sample was closed with a stopper and placed into the beaker for 2 mins, to ensure the majority of the yeast cells in the sample were killed, thereby forming the positive control for the FC method development. The remaining untreated sample was considered to be the negative control.

Prior to heat treating the cells, various other methods including ethanol treatment were experimented, the results can be found in appendix I.

3.1.2.5 Sample staining

To stain the yeast cells, the *Yeast Control - Viability kit* of *LOT number 230126* from *Sysmex* was used. The kit consisted of Solution A comprising of FDA and Solution B consisting of PI, which were used to stain the live and dead cells respectively. 200 μL of the 10^{-5} serial dilution of the sample containing the yeast cells was added per mL. Therefore, the cell concentration was estimated to be approximately 10^5 cells/mL, which was within the permissible cell density range of the flow cytometer. To this, 2 μL of Solution A was added and allowed to stain for 10 mins, per the staining instructions from the company. After 10 mins, 2 μL of Solution B was added. Post staining, the sample was vortexed and immediately analyzed using the flow cytometer. An initial dot plot was set up to distinguish cell size (FSC) and cell granularity (SSC), eliminating the background noise. Furthermore, the *Yeast Control - Viability kit* was used to create four quadrants which is elaborated in the results section 4.1.1.

The *Sysmex CyStain™ BacCount Viable* kit from *Sysmex* was used for staining the LAB cells. The kit comprised the dyes CyStain Green and CyStain Red which contain Thiazole Orange and PI respectively. 180 μL the serially diluted mash sample (10^{-4} dilution) was initially stained with 20 μL of CyStain Green and allowed to stain for 13 mins. After 13 mins, 2 μL of CyStain Red was later added to the sample and vortexed before after which it was immediately analyzed using the flow cytometer.

3.1.3 Spectrophotometry

Prior to FCM analysis, the absorbance of samples of different dilutions were measured using a *Hitachi U-2900 Double Beam Spectrophotometer* by loading cuvettes containing 1 mL of each sample into the spectrophotometer.

3.2 Colony counting

To analyze viable colonies, the active dry yeast was serially diluted in peptone water for colony counting. 4.5 mL of 0.1% peptone water was pipetted into 20 different test tubes and autoclaved using a *Tuttnauer Auto-steam sterilizer 2840EL - D* at 121°C for 15 mins. These solutions were later used to serially dilute 0.5 g of ADY up to 1:100,00 dilutions which were later used for streaking. To prepare the actual agar medium for incubation of the cell sample and thereby growth of yeast cell colonies, malt extract agar from *Merck KGaA, EMD Millipore corporation* was used. The agar was prepared using package

instructions; therefore 14.4 g of the Malt Extract Agar powder was dissolved in 300 mL of MQ water by gently inverting the Schott-Duran flask. Once the nutrient agar powder was dissolved completely, the now liquid malt extract agar medium was autoclaved before usage. The autoclaved agar was allowed to cool down to lukewarm temperature, after which an appropriate amount was poured into 15 different petri plates and allowed to solidify. Once solidified, dilutions ranging from 10^{-5} to 10^{-9} were streaked onto the petri plates and incubated at 37°C in a *WTC Binder - 78532* incubating oven *(Tuttlingen, Germany).* The plates were analyzed to identify which dilution provided a countable number of colonies and once estimated, the colonies were counted, and CFU/mL was calculated, and the results were compared to that of the FCM analysis.

3.3 Microscopy analysis

100 μL of the serially diluted sample (10^{-3}) was pipetted into an Eppendorf with methylene blue staining solution in a 1:1 ratio. This sample was vortexed well and 20 μL of this sample was pipetted into a *Burker Assistent haemocytometer*. The live and dead cell count was obtained manually using the *Nikon Eclipse 50i DS - Fi1* microscope from *LRi INstrument AB*. Figure 7a and 7b were captured using *DinoCapture 2.0 version 1.5.45*.

4. Results and discussion

4.1 Viability analysis of active dry yeast cells

Prior to flow cytometry analysis, the absorbance of samples with varying dilutions were assessed in order to estimate their absorbance. The reason for estimating the absorbance of several samples was to establish a fixed, ideal absorbance for Guava EasyCyte flow cytometer. The equipment at hand has a threshold for sample concentration and samples containing $>10^5$ concentrations of cells cannot be analyzed using this particular equipment. As absorbance directly correlates to concentration, estimating the optimal absorbance can help analyze mash samples of unknown cell concentrations in the future, by acting as a reference. Upon analyzing different dilutions, an average OD of 0.026 was found to be suitable for fermentation mash samples. The average absorbance of the remaining samples can be seen in appendix II.

The data obtained from the flow cytometry analysis was further analyzed in order to establish a coherent method for optimal viability analysis of *Saccharomyces cerevisiae* and *Lacticaseibacillus casei* during fermentation.

4.1.1 Flow cytometry analysis of pure active dry *Saccharomyces cerevisiae*

While multiple studies have previously utilized FCM to analyze various physiological aspects of yeast cells successfully for decades, this step forms the basis of analysis of yeast from mash samples and

therefore cannot be evaded. In order to separate the yeast cells from background "noise" or particles which are not considered to be yeast cells, a Forward Scatter (FSC-H) vs. Side Scatter (SSC-H) plot was set up. To separate the desired events more clearly from the undesired background, a threshold of 240 was set on the FSC-H, as the events counted up to this threshold were entirely unnecessary noise. The equipment was set to analyze up to 2000 events per run and the Yeast Control viability kit containing FDA and PI was used for analysis. As the yeast cells are comparatively much larger in size, post these instrument settings, a clear separation between the cells and the background was obtained, as can be seen in figure 3. In this case, the yeast cells were easily distinguishable from the background noise due to their increased cell size and granularity.

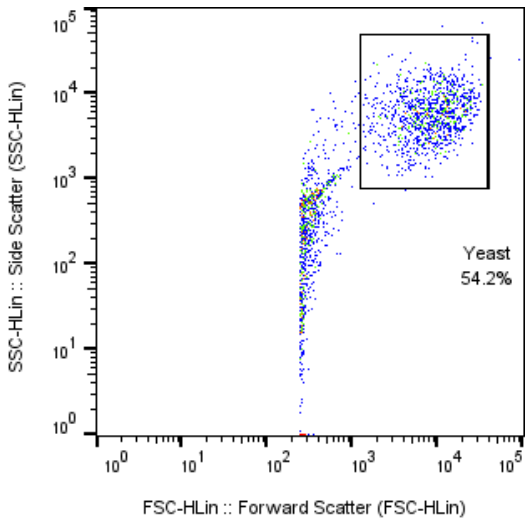

Figure 3: Logarithmic FC dot plot showing a gated population *Saccharomyces cerevisiae* analyzed from a serialy diluted (10^{-5}) sample of rehydrated, freeze-dried, active dry yeast.

Post identifying the yeast cells, they were gated onto an FL1 vs. FL3 plot to obtain information regarding their viability status. Four different quadrants were set while working with the kit containing a combination of FDA and PI (Yeast Control Viability kit) so as to quickly estimate the viability level of the population present in each quadrant (see figure 4). To efficiently identify and estimate the positions of the live, dead, and damaged cell populations, both untreated and heat-treated samples were used. While these quadrants helped assess yeast cells, they were established to be inaccurate in samples containing combined microorganisms. To fully understand the effects of the dyes utilized, the serially diluted sample was assessed as is, and later heat-treated for 2 mins at ~90°C several times without the dye to understand the yeast autofluorescence, and with FDA and PI both individually and combined. Additionally, plots to discriminate doublets and fluidics vs. time plots were utilized to provide information on run stability were also set up in order to maximize accuracy of the final results obtained.

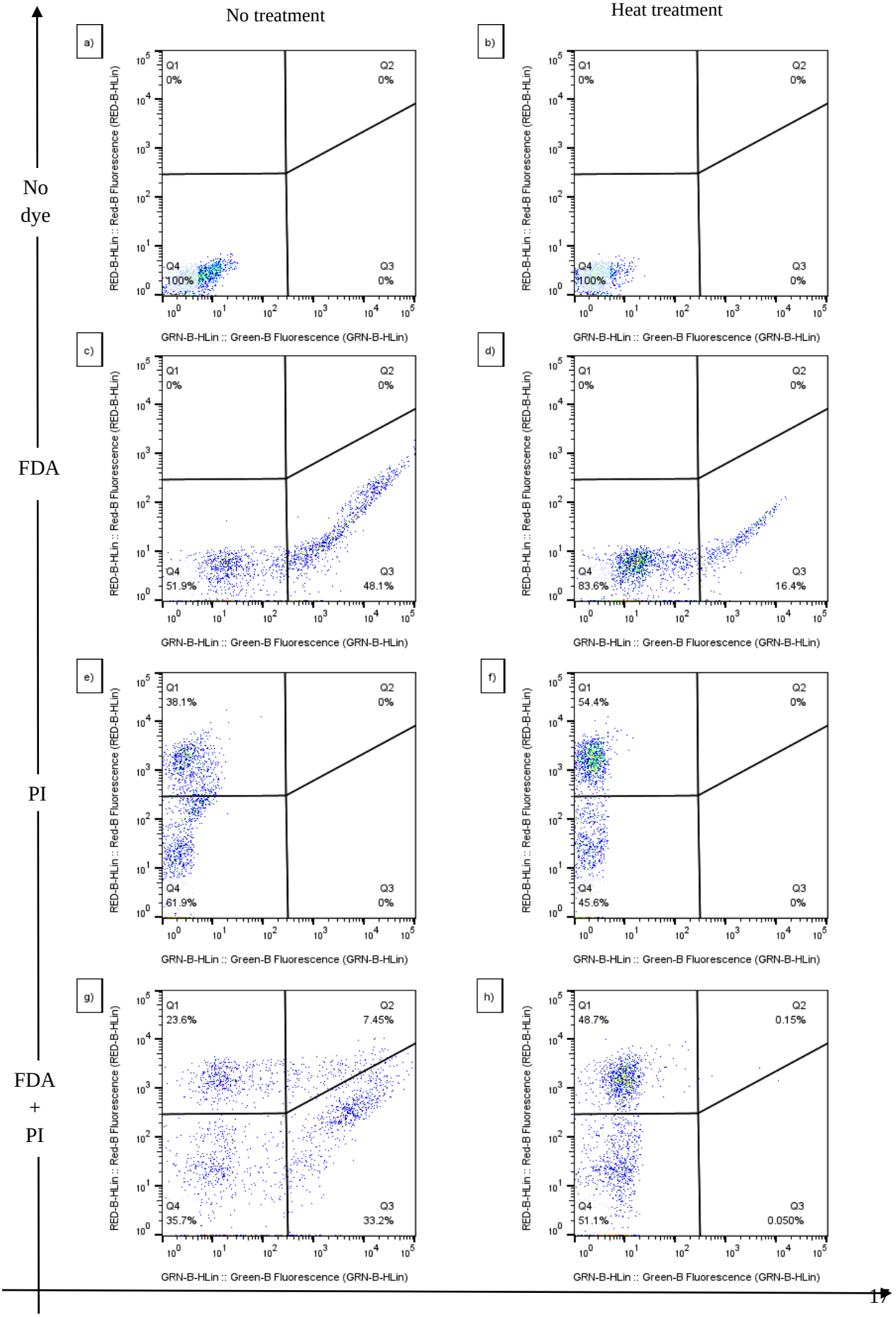
No treatment
Heat treatment
No dye
FDA
PI
FDA + PI
a)
b)
c)
d)
e)
f)
g)
h)
Q1 0%
Q2 0%
Q4 100%
Q3 0%
Q1 0%
Q2 0%
Q4 100%
Q3 0%
Q1 0%
Q2 0%
Q4 51.9%
Q3 48.1%
Q1 0%
Q2 0%
Q4 83.6%
Q3 16.4%
Q1 38.1%
Q2 0%
Q4 61.9%
Q3 0%
Q1 54.4%
Q2 0%
Q4 45.6%
Q3 0%
Q1 23.6%
Q2 7.45%
Q4 35.7%
Q3 33.2%
Q1 48.7%
Q2 0.15%
Q4 51.1%
Q3 0.050%
RED-B-HLin :: Red-B Fluorescence (RED-B-HLin)
GRN-B-HLin :: Green-B Fluorescence (GRN-B-HLin)

Figure 4: All 8 FC dot plots shown above are logarithmic FL1 vs. FL3. The quadrants were defined as follows: upper left (Q1) – dead cells, upper right (Q2) – damaged cells, lower left (Q4) – quiescent or dormant cells, lower right (Q3) - metabolically active or live cells. The samples were stained with the dyes FDA and PI. Dot plots a., c., e., and g. represent untreated samples stained with no dye, only FDA, only PI, and FDA + PI respectively. Dot plots b., d., f., and h. represent heat- treated samples stained with no dye, only FDA, only PI, and FDA + PI respectively.

It is interesting to note that despite the absence of heat treatment, the cells showed a high number of dead cells (23.6%) (Figure 4g). This could be attributed to the following speculated reasons: the yeast cells used at TAC (The Absolut Company) during the course of the project have also been stored at room temperature for a period of time which may play a pivotal role in the reduced viability of yeast cells. Moreover, the usage of Rehydrated Freeze-Dried Yeast (RFDY) cells can also play a crucial role. Yeast cells obtained from a pure culture can vary greatly in terms of viability from rehydrated freeze-dried yeast cells due to the shock that the latter have been exposed to.

a) b)

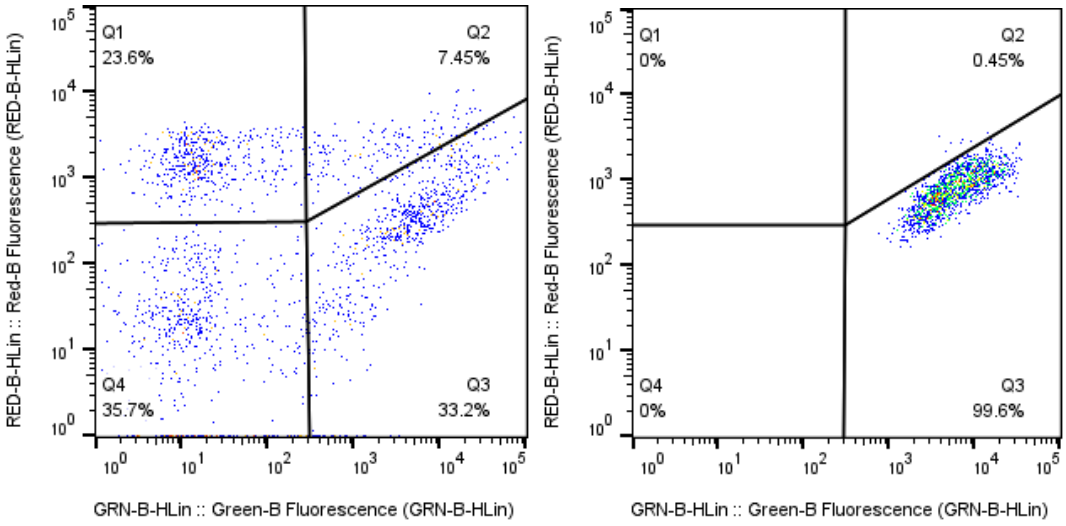

Figure 5: **a)** FCM analysis of active dry yeast in a logarithmic dot plot, and **b)** FCM analysis cultured yeast cells in a logarithmic dot plot.

Figure 5 shows a comparison of FCM analyses of active dry yeast (figure 5a) and cultured yeast (figure 5b) respectively. While the active dry yeast display yeast cells of varying viability, only 33.2% are found to be completely metabolically active while cultured yeast cells portray an overwhelmingly alive (99.6%) yeast cell population. This further reinstates the effect that the previously speculated reasons could have on yeast cells. It was also noted that the live cells present in quadrant Q3 while emitting a high green fluorescence, as expected, also seem to emit a high red fluorescence. This could be attributed to the ability of a cell to exclude PI itself, as this phenomenon can be considered to be directly proportional to the cell's viability level.

Total yeast cells				Live yeast cells				Dead yeast cells			
Count	**Conc.**	**Percent.**	**Avg. log**	**Count**	**Conc.**	**Percent.**	**Avg. log**	**Count**	**Conc.**	**Percent.**	**Avg. log**
1111	2.16E+10	100%	10.33	420	8.11E+09	37.87%	9.91	355	6.84E+09	31.99%	9.83

Table 2: Average cell counts, concentrations, and percentages of gated live, dead, and total yeast cell populations obtained from an untreated, serially diluted, active dry yeast sample of 10^{-5} dilution.

It is important to note that the damaged cells have been considered in the total cell count. The results obtained from rapid FC analysis were also comparable to other conventional viability assessment techniques, as can be seen in later sections of the results.

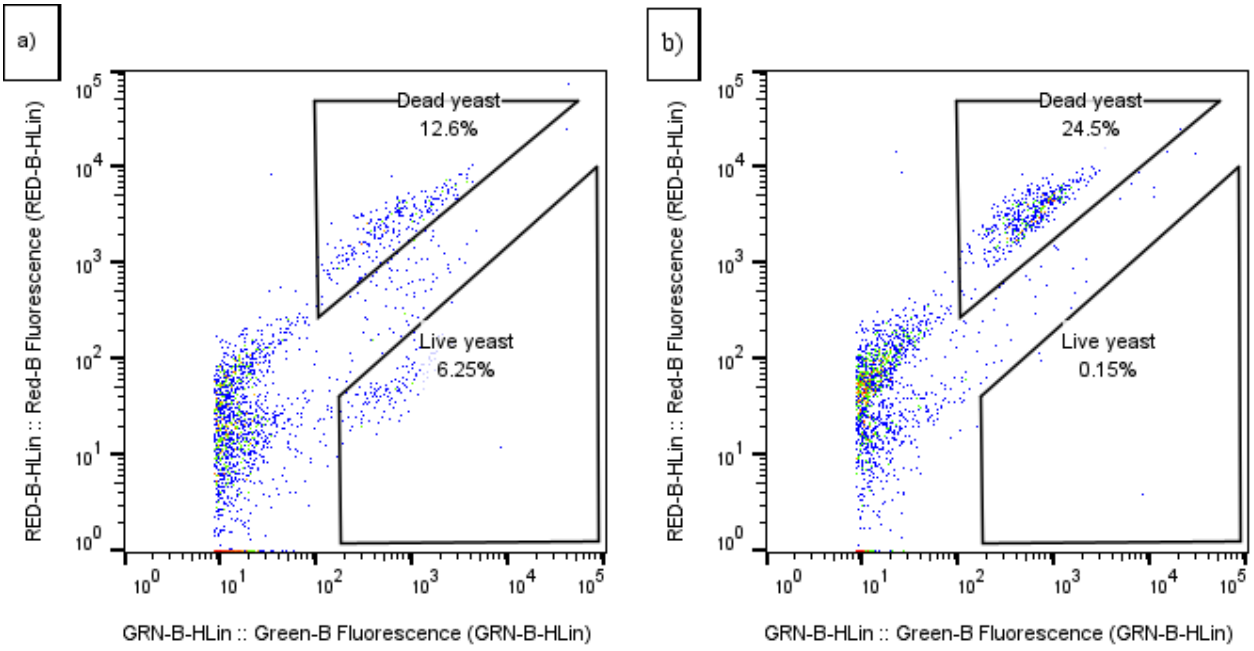

Figure 6: a) Logarithmic FC dot plot of an untreated sample of *Saccharomyces cerevisiae*, serially diluted with water and stained using thiazole orange and PI. **b)** Logarithmic FC dot plot of an untreated sample of *Saccharomyces cerevisiae*, serially diluted with water and stained using thiazole orange and PI.

Finally, the rehydrated, freeze-dried yeast was also analyzed with the BacCount kit containing TO and PI. This was done to create and set a template for yeast when stained with TO instead of FDA, as it would be in with the mash samples in the future. As seen in figure 6, the BacCount kit while providing cell viability information for the untreated and heat-treated samples of yeast, the information is limited to the live or dead cell status, unlike FDA which was able to also analyze whether the cell was damaged or quiescent.

4.1.2 Colony counting of serially diluted *Saccharomyces cerevisiae*

Colony counting was one of the parallel methods that was performed to validate and compare the results obtained from FCM analysis. Moreover, establishing the suitable dilution of samples that can be used for plate streaking provides a reference for future experiments conducted at The Absolut Company. Post incubation and analysis of various dilutions ranging from 10^{-5} to $10^{-}9$, the 10^{-7} dilution had an average of 102.66 colonies. This results in a concentration of $1.02*10^{10}$ CFU/mL and is considered to be a similar result to FCM as P>0.05 i.e., P = 0.0663 from which we can conclude that there is no statistical difference between the two analysis methods. Therefore, the 10^{-7} dilution was established to be best suited for colony counting of Active Dry Yeast cells. Refer to Appendix VI to see results from the colony counting of dilutions 10^{-5} to 10^{-9}.

4.1.3 Haemocytometer microscopy analysis to determine yeast cell viability

The last parallel technique performed in order to compare the FCM results was microscopy analysis with the help of a haemocytometer. Methylene blue was used to stain the dead cells blue while the live cells remained colorless (see figure 7). Post analysis, an average total yeast cell concentration of 1.46E+10 cells /mL was obtained. The average live and dead yeast cell concentrations were calculated to be 7.72E+09 cells/mL and 3.31E+09 cells/mL respectively. As P>0.05 (P = 0.2022), the haemocytometer microscopy analytical technique for yeast cell viability analysis was concluded to have no statistical difference when compared to FCM. As seen in figure 6, the dead (blue) yeast cells appear to be smaller than the colorless live cells. During the various FCM analyses of yeast performed throughout the course of the project, it was also observed that the dead yeast cells had a noticeably smaller FSC and SSC pointing to their reduced cell size and granularity. This phenomenon could be explained as temperature plays a vital role in the average diameter of the yeast cells. For instance, the average diameter of single yeast cells was estimated to be 7.94 µm (Zakhartsev, & Reuss, 2018) at temperature between 18.5°C and 40°C, but when the temperature was lowered from 18.5°C, down to 5°C, an exponential increase in the average cell diameter to 10.2 µm was observed.

a) b)

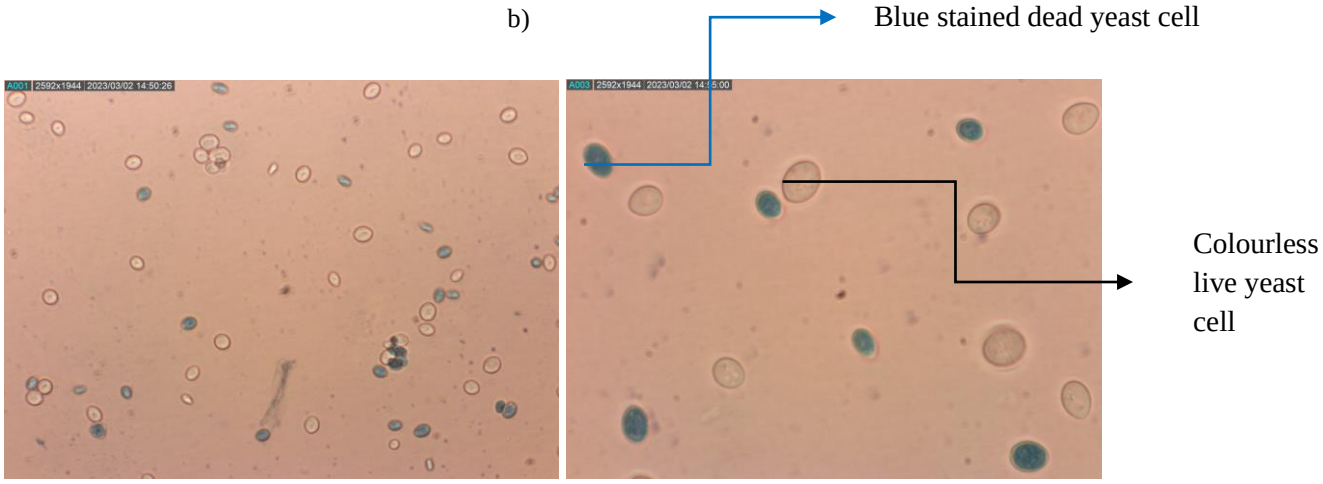

Figure 7: a) a 40x magnified image of yeast cells where the live cells are colorless, and the dead cells are stained blue with methylene blue **b)** a 100x magnified image of yeast cells stained with methylene blue.

Based on the results obtained from the analysis of ADY by several different methods of viability analysis, the results could be summarized as follows:

Analytical method	Total (cells/mL)	Live (cells/mL)	Dead (cells/mL)
Microscopy	1.46E+10	7.72E+09	3.31E+09
FCM	2.16E+10	8.11E+09	6.84E+09
Colony Forming Unit	n.a.	1.02E+10	n.a.

Table 4: Average cell concentrations of live, dead, and total yeast cell populations as obtained from viability analytical techniques such as microscopy, FCM and colony counting analysis.

The absence of a statistically significant difference in the cases of both colony forming unit technique as well as haemocytometer microscopy analysis when compared to the FCM analysis in the total yeast cell concentrations reiterates the reproducibility and robust nature of the results obtained over the course of the project using the Flow cytometry analytical technique.

4.2 Flow cytometry analysis of *Lacticaseibacillus casei*

While LAB cells are not uncommon in alcoholic fermentation, there has been no prior research undertaken to quantify or analyze them in vodka fermentations. The concerns that arise with the analysis of lactic acid bateria via flow cytometry are predominantly two fold. The size of lactic acid bacteria (0.7 to 1 µm) (Schär-Zammaretti, & Ubbink, 2003) and the size of yeast (5 – 6 µm) (Yeasts, n.d.) are significantly different. This results in yeast cells being much easier to distinguish from the background cell debris than lactic acid bacteria due to their marked separation from the noise (as explained in section 4.1.1). the second concern is the concentration of LAB that occurs naturally in alcoholic fermentations. For this project, *Lacticaseibacillus casei* cells have been added to mash samples so as to assess the efficiency of the method that's beinf developed. When compared to yeast cells, the naturally occuring lactic acid bacteria in fermentation can be as much as 100 times lower (Rodriguez and Thornton, 2007). Viability analysis of *Lacticaseibacillus casei* cells without the addition of yeast, present in the fermentation mash samples was initially performed. While the yeast cells displayed a clear separation due to the above mentioned reasons, the major challenge during this method development was to identify a qay to isolate, quantify, and later analyze the LAB cells from the mash sample. It is important to note that the mash samples used in this project are only treated with enzymes and are not from the fermentation tanks of The Absolut Company).

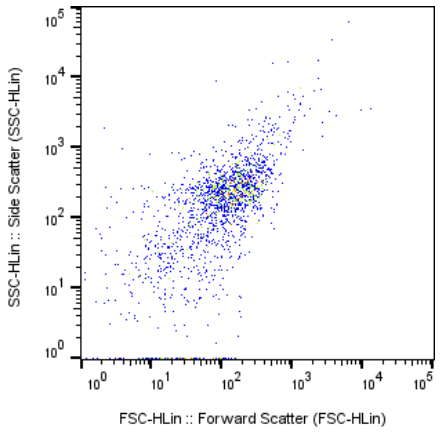

Figure 8: FSC vs. SSC FC dot plot of LAB cells analyzed from fermentation mash sample showing no distinguishable characteristics between backgrounf noise and LAB events.

As seen in figure 8, the *Lacticaseibacillus casei* cells appear to be combined with the background noise when analyzed in an FSC vs. SSC dot plot., as done for the yeast cells. In order to isolate the actual bacterial cells, the sample was initially directly analyzed in an FL1 vs FL3 dot plot. In order to ensure that the events measured by the flow cytometer were predominantly baterial cells and not particles from the background mash, a suitable threshold was estimated. Untreated and heat-treated acterial-mash samples were analyzed at thresholds starting from 0, 10, 20, 30 and 40 (see figure 9) to analyze the relationship between the threshold, total cell percentage and cell debris.

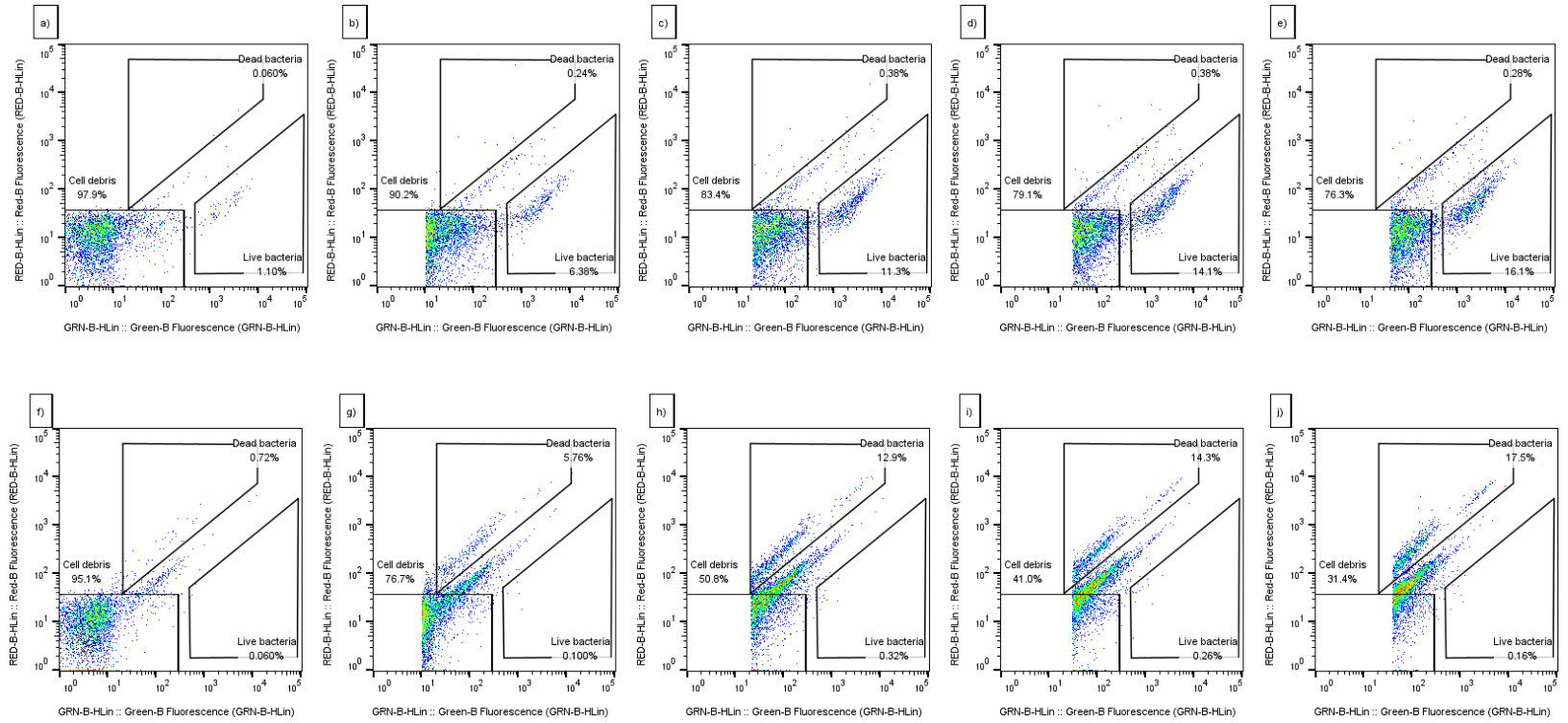

Figure 9: Dot plots a) to e) represent untreated samples starting from threshold 0, 10, 20, 30, and 40 respectively. Dot plots f) to j) represent heat-treated samples starting from threshold 0, 10, 20, 30, and 40 from f) to j) respectively.

Figure 8a displays a logarithmic FC dot plot of a mash sample containing *Lacticaseibacillus casei* cells. The flow cytometer was set to acquire 5000 events and it is evident that a majority (97.90%) of these events are comrpised of the comparitively bigger mash particles. By increasing the threshold, the flow cytometer was set to ignore larger mash particles and provide more information regarding the *Lacticaseibacillus casei* cells, as can be seen in figure 9e which is an untreated sample consisting of 16.1% live LAB cells. While the background noise cannot be completely evaded, it was estimated that a threshold of 40 was most suitable for the samples at hand. This is because, an increased threshold provides more accurate counts for metabolically active *Lacticaseibacillus casei* cells, also limited the informaton that could be acquired for the dead *Lacticaseibacillus casei* cells. This is due to the reduced green fluorescence provided by the dead cells which compromises the accuracy of the information obtained as the threshold is set as a function of the green fluorescence. As encountered with the yeast cells, heat-treatement and thereby death of both the yeast and bacterial cells reduced their FSC and SSC. With these factors taken into consideration, a threshold of 40 was estimated to be most suitable for isolating and quantifying *Lacticaseibacillus casei* cells in a sample diluted with mash. An inversely proportional relationship between the total cell percentage and the cell debris was observed with an increase in threshold, this is visualized in the graph provided in figure 10.

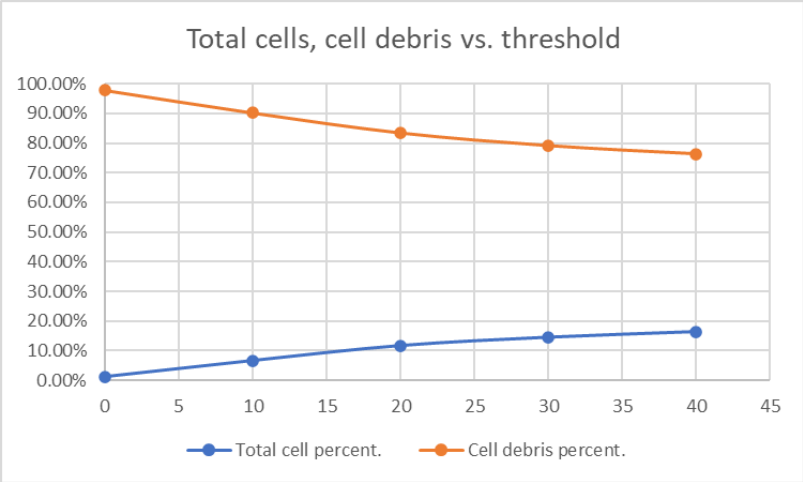

Figure 10: Graph depicting the increase in threshold resulting in the increase of total cell percentages and decrease in cell debris. The sample analyzed was an untreated mash sample containing *Lacticaseibacillus casei* cells.

Post incorporating these settings to acquire results of highest possible accuracy for the equipment and conditions provided gates were set using the analyzed control samples (see figure 11) where untreated and heat-treated (2 mins at ~90°C) *Lacticaseibacillus casei* containing samples were serially diluted in enzyme-treated fermentation mash. As seen in the figure 11b, the live bacteria now have an increased red fluorescence indicating cell damage or death, as expected of the heat treatment.

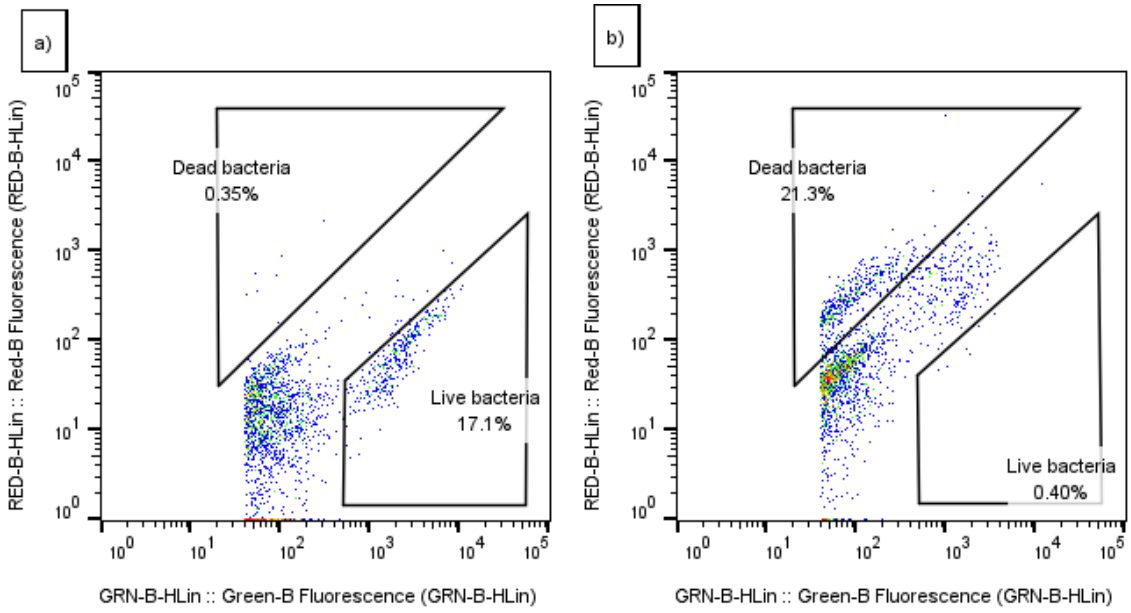

Figure 11: a) Logarithmic FC dot plot of an untreated sample of *Lacticaseibacillus casei*, serially diluted with enzyme-treated mash and stained using thiazole orange and PI. **b)** Logarithmic FC dot plot of an untreated sample of *Lacticaseibacillus casei*, serially diluted with enzyme-treated mash and stained using thiazole orange and PI.

The live and dead bacteria seen in figures 11a and 11b respectively have a reasonable separation from the background noise, which was absent before the increase in threshold from 0 to 40. Prior to heat-treatment, no clear separation of the dead cells from the background can be seen, but post heat-treatment, the reduction in cells in the "Live bacteria" gate from 17.1% to 0.40% and increase in the number of events in the "Dead bacteria" gate from 0.35% to 21.3%, allow the clear quantification and working of the viability analyses of *Lacticaseibacillus casei.*

Total cells				Live cells				Dead cells			
Count	**Conc.**	**Percent.**	**Avg. log**	**Count**	**Conc.**	**Percent.**	**Avg. log**	**Count**	**Conc.**	**Percent.**	**Avg. log**
679	2.08E+09	100	9.32	341	1.05E+09	50.22	9.02	338	1.04E+09	49.77	9.01

Table 3: Average cell counts, concentrations, and percentages of gated live, dead, and total cell populations of an untreated, serially diluted *Lacticaseibacillus casei* cell sample.

In table 3, it is important to note that the damaged LAB cells have not been considered in the total cell count. In order to get accurate data regarding the number of cells present in the sample, the background noise and damaged cells present in the FC dot plot have not been considered in the viability calculations. That is, the total number of cells has been assumed to be 100% of the cells present in the sample. The sample analyzed was not incubated for any period of time and directly analyzed, despite this, the live and dead cell percentages was calculated to be nearly equal which could be attributed to the usage of a relatively old sample of bacterial suspension which was used, despite refrigeration.

4.3 Flow cytometry analysis of yeast-bacteria mixed cultures

The main aim of this project was to understand, isolate, quantify and analyze the microbial diversity (mainly *Saccharomyces cerevisiae* and *Lacticaseibacillus casei*) present in the fermentation broth of alcoholic vodka fermentations. The following FC dot plots were set up in order to simultaneously quantify and analyze the desire cell populations:

1. FSC vs. SSC (to separate yeast cells from background noise)
2. FL1 vs. FL3 (to separate live yeast cells, dead yeast cells and damaged yeast cells)
3. FL1 vs. FL3 (to separate live yeast and bacterial cells from dead yeast and bacterial cells)
4. FL1 vs. SSC (to separate live yeast from live LAB)
5. FL1 vs. SSC (to separate dead yeast from dead LAB)

4.3.1 Gating strategy for *Saccharomyces cerevisiae*

In the case of Active Dry Yeast, as mentioned in results sections 4.1.1. The separation was evident due to the difference in the yeast cell size and size of the background noise. This separation can clearly be seen in figure 12a displayed in the FSC vs. SSC plot. The yeast cells obtained from the FSC vs. SSC plot were

further gated onto an FL1 vs. FL3 plot (figure 12b). The template that was created while staining the active dry yeast cells with the BacCount kit was used for analysis of ADY in mash samples.

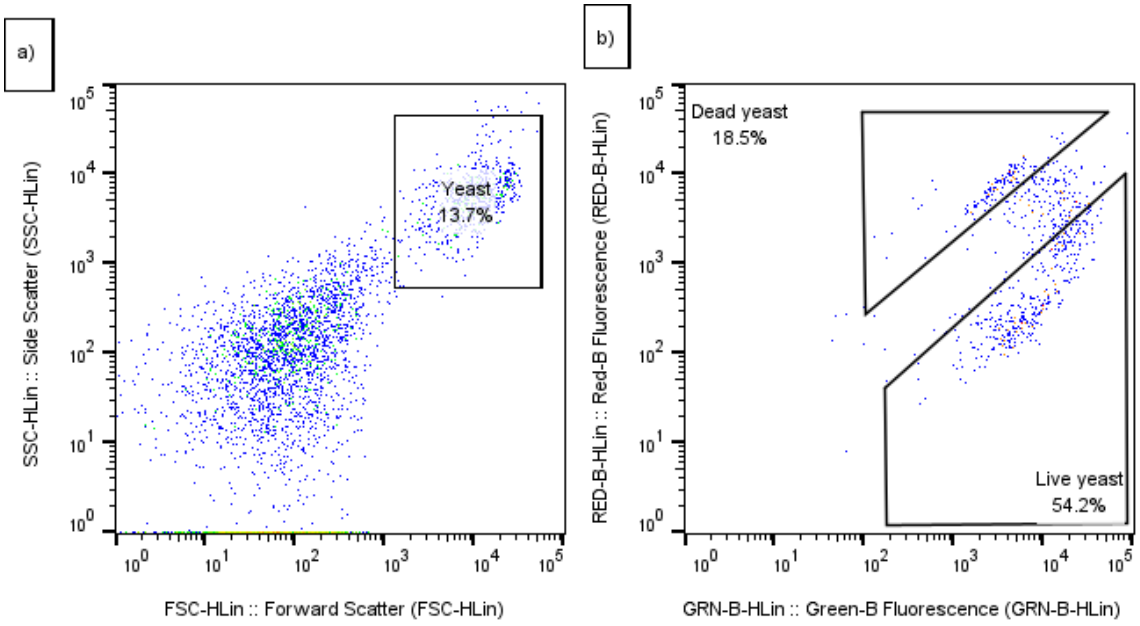

Figure 12: a) Logarithmic FC dot plot (FSC vs. SSC) of a serially diluted mash sample (0 hours) containing both yeast and bacteria with a clear separation between the yeast cells (within the parent gate) and background noise **b)** Logarithmic FC dot plot (FL1 vs. FL3) showing live and dead yeast cells gated from the parent yeast gate.

4.3.2 Gating strategy for *Lacticaseibacillus casei*

The gating strategy involved in isolating and quantifying the LAB was done by setting up an FL1 vs. FL3 plot as seen in figure 7, contrary to ADY. This was done due to the immense size difference between the yeast and LAB cells. While this size difference aided in distinguishing the yeast and bacterial populations, the challenge lay in separating the LAB cells from the mash particulates which, when viewed in a FSC vs. SSC plot, appeared to be indistinguishable. To overcome this issue, the FSC vs. SSC plot was neglected and the sample was initially directly analyzed in a FL1 vs. FL3 FC dot plot, as seen in figure 13a. The live and dead gates contain both yeast and LAB cells and which were further gated on two separate FL1 vs. SSC dot plots so as to obtain a clear separation between the live yeast and bacterial cells (figure 13b) and the dead yeast and bacterial cells (figure 13c). The cell counts, concentrations, and percentages were calculated and can be seen in appendix V b.

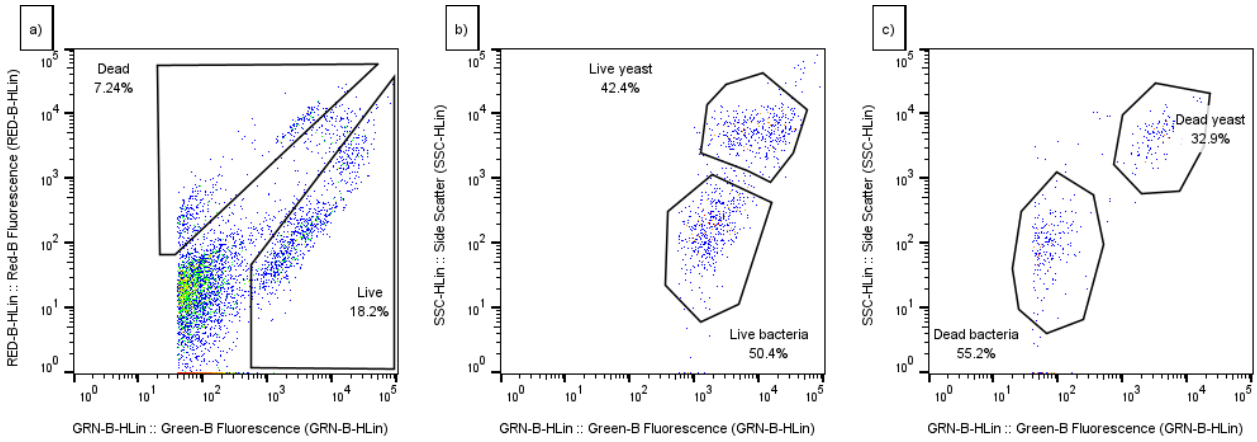

Figure 13: a) FL1 vs. FL3 FC dot plot showing the live and dead cells present in fermentation mash sample, **b)** FL1 vs. SSC FC dot plot showing separation between the live yeast and LAB cells due to the difference between the granularity of the two populations, **c)** FL1 vs. SSC FC dot plot showing separation between the dead yeast and LAB cells due to the difference between the granularity of the two populations.

Viability analyses for yeast and LAB were conducted via FCM at 0, 24, 48-hour intervals in order to understand microbial interactions in vodka fermentations at a laboratory scale, showing a steady decline in the live cell population and a steady increase in the dead cell population over time (figure 14). Moreover, the 48-hour period analysis provides a real-time representation of the mash sample with an increase in debris over time indicating the gradual degradation of mash by the yeast and bacteria over time. The decrease in the clear separation of live and dead events over time can be an indicator of a large number of cells being damaged and present in a more in between level of viability, thereby not fitting under a strict live or dead gate in FCM analysis. The cell concentrations were calculated, and the microbial growth curves have been displayed in figure 15.

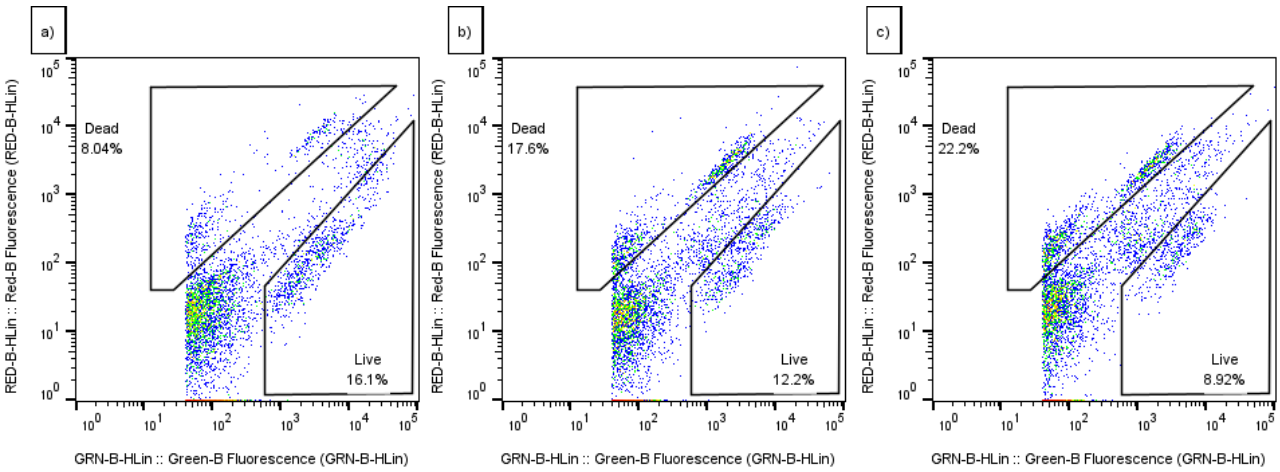

Figure 14: a) Logarithmic FC dot plot of an enzyme-treated mash sample containing added populations of *Saccharomyces cerevisiae* and *Lacticaseibacillus casei* at 0 hours, **b)** Logarithmic FC dot plot of an enzyme-treated mash sample containing added populations of *Saccharomyces cerevisiae* and *Lacticaseibacillus casei* at 24 hours, **c)** Logarithmic FC dot plot of an enzyme-treated mash sample containing added populations of *Saccharomyces cerevisiae* and *Lacticaseibacillus casei* at 48 hours.

Contrary to a typical growth curve, the live cell count was found to reduce with increase in time. This is attributed to the fact that while the mash sample containing water, wheat flour and enzymes were used, the specific samples used for FCM analysis were not obtained from the large-scale TAC fermenters, rather from laboratory scale fermentation performed for this project with a known amount of RFDY and LAB sample added to the mash. A large-scale fermenter would provide optimal growth conditions for the microorganisms, including constant agitation, controlled temperatures etc. which was not provided for the

samples included in the mash analysis. This could be a speculated reason resulting in the decline of overall live cells and an increase in the overall dead cells.

TAC currently utilizes HPLC to determine the increase in organic acids which correlates to the increase in LAB which indicates the fermentation tanks need to be cleaned. The usage of FCM in analyzing mash samples can result in the estimation of a LAB count, which when exceeded could indicate the need to clean the fermenters. While HPLC is an efficient way to gather information regarding the composition of the sample, incorporation of FCM could greatly save time and even the amount of sample required to arrive on an almost immediate status update regarding whether or not the tanks need to be cleaned. Although initially, a range for permissible LAB concentrations correlating to amount of organic acids present would need to be set up.

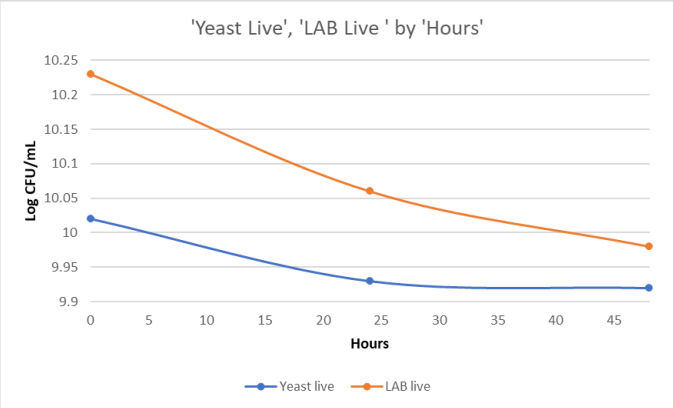

Figure 15: Graph depicting decline in *Lacticaseibacillus casei* over a 48-hour incubation period.

5. Conclusion

To summarize the results and findings obtained throughout the course of this project, existing microbial viability analytical techniques such as microscopy analysis and colony counting are subject to a number of drawbacks. The combined cons of these techniques and the unique pros of flow cytometry have made it a suitable candidate for the simultaneous analysis of multiple microorganisms, quantitatively. This is the purpose of utilizing FCM for the first time in the viability analyses of the microbial diversity of alcohol fermentations, at The Absolut Company, Åhus. While the final result (Yeast-Bacteria growth curve) obtained and presented in the report seem contradictory to a typical microbial growth curve, it is essential to remember that the growth conditions presented to the microbes were subpar and the pivotal aim of this project was to develop a flow cytometry analytical technique to quantify and analyze *Saccharomyces cerevisiae* and lactic acid bacteria, which has been accomplished.

The developed method can not only be applied in rapidly identifying and quantifying the desired microbial populations such as yeast, but also be used to track the viability of the microorganism over the course of the fermentation period and provide valuable input regarding the final product. The developed method also helps overcome the challenge of identifying the desired microorganisms from a complex matrix which might otherwise pose a substantial hindrance to existing analytical techniques. While there is definitely room for improvement in the quantification and analysis of lactic acid bacteria from mash samples, this project forms the basis providing a new perspective on the microbial interactions that occur during alcohol fermentation.

As mentioned in the report, the viability analyses of yeast and LAB conducted over the course of the project were not performed on the mash samples from the fermentation tanks of The Absolut Company. Analyzing the mash samples from directly from the fermentation tanks could be a way to start FCM process optimization by fine tuning the existing Yeast-LAB template or creating multiple time specific templates to suit the samples collected at different time intervals. Future effort can also be dedicated to combining flow cytometry with other available online resources so as to be able to analyze the fermentation samples periodically and automatically at regular time intervals. This technique could provide valuable information regarding yeast, LAB, and possibly quantify the other microorganisms that are able to thrive during fermentation.

Another way to optimize the existing FCM technique could be the incorporation of fluorescently labelled antibodies to tag the yeast, LAB, and potentially other microorganisms present in the fermentation tanks so as to better understand their actions and interactions alcohol fermentation. For instance, with the usage of FCM the yeast cells tend to be present as one population in the dot plots. Incorporation of a fluorescent yeast antibody could help distinguish between different *Saccharomyces cerevisiae* strains for more detailed research and analyses.

6. References

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Appendices

I. Results from ethanol treatment

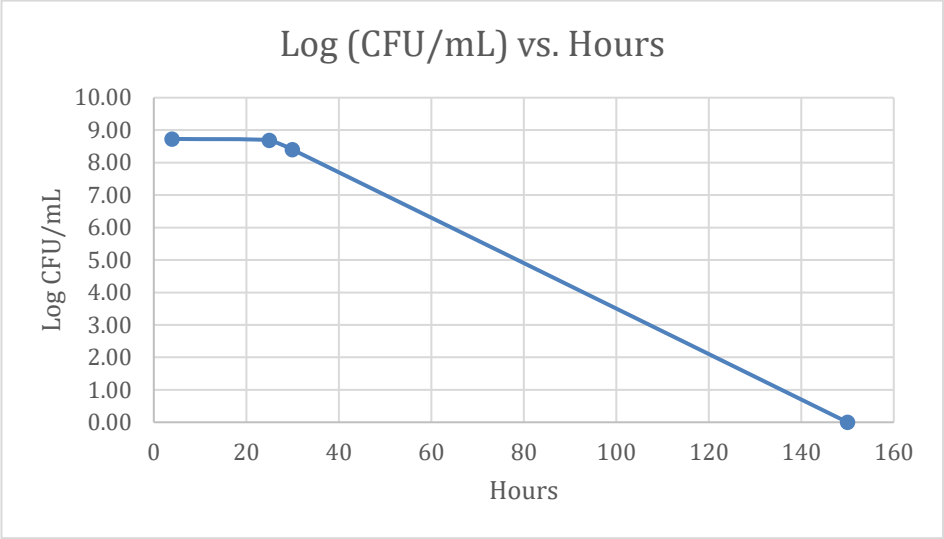

Figure 1: Reduction in viable *Saccharomyces cerevisiae* cells over time when present in a sample of 40% ethanol.

II. Spectrophotometry

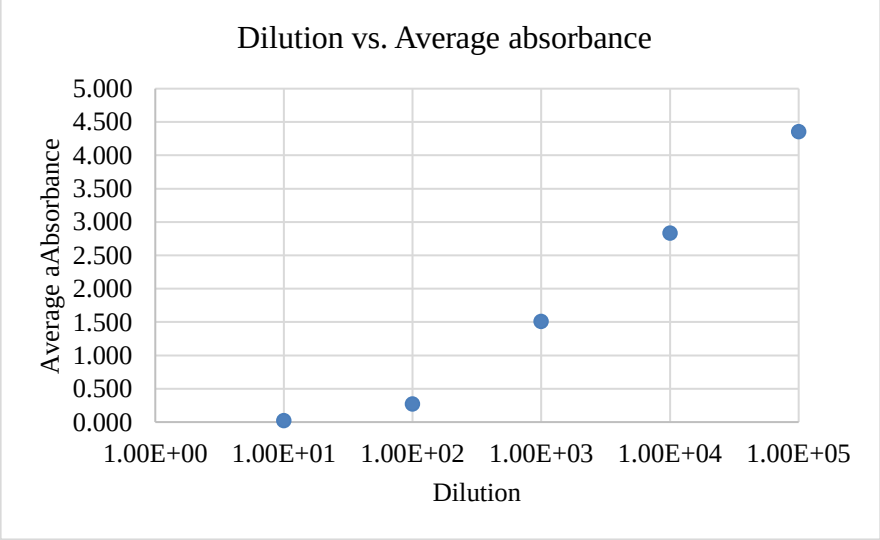

Figure 1: Graph depicting samples of varying dilution plotted against their respective average absorbance measured via spectrophotometry.

III. ADY analysis raw data

Total yeast cells				Live yeast cells				Dead yeast cells			
Count	Conc.	Percent.	Log	Count	Conc.	Percent.	Log	Count	Conc.	Percent.	Log
1037	2.33E+10	100	10.37	412	9.04E+09	39.73	9.96	330	7.24E+09	31.82	9.86
1150	2.08E+10	100	10.32	447	8.08E+09	38.87	9.91	349	6.31E+09	30.35	9.80
1145	2.06E+10	100	10.31	401	7.22E+09	35.02	9.86	387	6.96E+09	33.80	9.84
1111	**2.16E+10**	**100**	**10.33**	**420**	**8.11E+09**	**37.87**	**9.91**	**355**	**6.84E+09**	**31.99**	**9.83**

IV. LAB analysis raw data

Total cells				Live cells				Dead cells			
Count	Conc.	Percent.	Log	Count	Conc.	Percent.	Log	Count	Conc.	Percent.	Log
712	2.03E+09	100	9.31	323	9.21E+08	45.37	8.96	389	1.11E+09	54.63	9.05
690	2.04E+09	100	9.31	363	1.08E+09	52.61	9.03	327	9.69E+08	47.39	8.99
636	2.18E+09	100	9.34	337	1.16E+09	52.99	9.06	299	1.03E+09	47.01	9.01
679	**2.08E+09**	**100**	**9.32**	**341**	**1.05E+09**	**50.32**	**9.02**	**338**	**1.04E+09**	**49.68**	**9.01**

V. ADY-LAB analysis raw data

a) Yeast:

Hours	Total						Live						Dead					
	Count	%	Conc.	Log	Avg. Log	Stdv	Count	Conc.	%	Log	Avg. Log	Stdv	Count	Conc.	%	Log	Avg. Log.	Stdv
0	699	13.98%	1.97E+10	10.29	10.30	0.01	414	1.17E+10	59.23%	10.07	10.02	0.04	181	5.10E+09	25.89%	9.71	9.80	0.07
	735	14.70%	2.10E+10	10.32			364	1.04E+10	49.52%	10.02			254	7.25E+09	34.56%	9.86		
	728	14.56%	1.95E+10	10.29			353	9.45E+09	48.49%	9.98			252	6.75E+09	34.62%	9.83		
24	1045	20.90%	2.26E+10	10.35	10.35	0.01	401	8.66E+09	38.37%	9.94	9.93	0.01	499	1.08E+09	47.75%	9.03	9.71	0.48
	1109	22.18%	2.18E+10	10.34			433	8.50E+09	39.04%	9.93			541	1.06E+10	48.78%	10.03		
	1217	24.34%	2.21E+10	10.34			545	8.24E+09	37.30%	9.92			629	1.14E+10	51.68%	10.06		
48	1089	21.78%	2.25E+10	10.35	10.37	0.01	421	8.68E+09	38.66%	9.94	9.92	0.03	498	1.03E+10	45.73%	10.01	10.06	0.03
	1225	24.50%	2.42E+10	10.38			446	8.81E+09	36.41%	9.94			581	1.15E+10	47.43%	10.06		
	1331	26.62%	2.35E+10	10.37			434	7.67E+09	32.61%	9.88			704	1.24E+10	52.89%	10.09		

b) Bacteria

Hours	Total						Live						Dead					
	Count	Conc.	%	Log	Avg. log	Stdv	Count	Conc.	%	Log	Avg. Log	Stdv	Count	Conc.	%	Log	Avg log	Stdv
0	3195	9.01E+09	63.90%	9.95	9.95	0.01	589	1.66E+10	11.78%	10.22	10.23	0.01	2606	7.35E+10	52.12%	10.87	10.86	0.01
	3188	9.09E+09	63.76%	9.96			613	1.75E+10	12.26%	10.24			2575	7.35E+10	51.50%	10.87		
	3217	8.62E+09	64.34%	9.94			613	1.64E+10	12.26%	10.21			2604	6.97E+10	52.08%	10.84		
24	2932	6.33E+09	58.64%	9.80	9.76	0.04	570	1.23E+10	11.40%	10.09	10.06	0.03	2362	5.10E+10	47.24%	10.71	10.66	0.04
	2897	5.69E+09	57.94%	9.76			598	1.17E+10	11.96%	10.07			2299	4.51E+10	45.98%	10.65		
	2830	5.13E+09	56.60%	9.71			569	1.03E+10	11.38%	10.01			2261	4.10E+10	45.22%	10.61		
48	3010	6.21E+09	60.20%	9.79	9.75	0.04	536	1.11E+10	10.72%	10.05	9.98	0.05	2474	5.10E+10	49.48%	10.71	10.66	0.04
	2875	5.68E+09	57.50%	9.75			468	9.24E+09	9.36%	9.97			2407	4.75E+10	48.14%	10.68		
	2782	4.92E+09	55.64%	9.69			478	8.45E+09	9.56%	9.93			2304	4.07E+10	46.08%	10.61		

VI. Colony counting

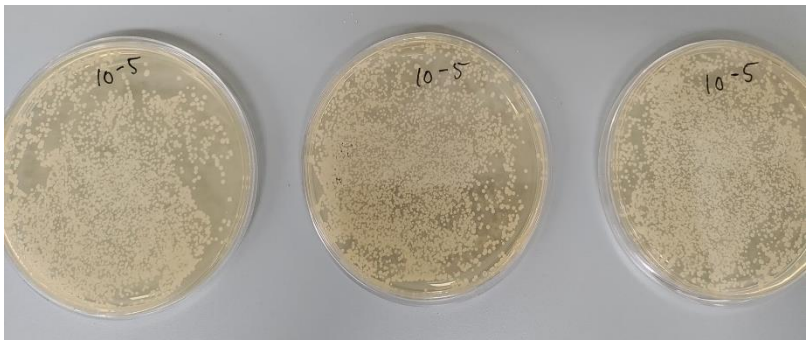

Figure 1: Colonies grown from the streaking of a serially diluted active dry yeast sample of 10^{-5} dilution, streaked on three malt extract agar plates. The colonies were considered too numerous to count.

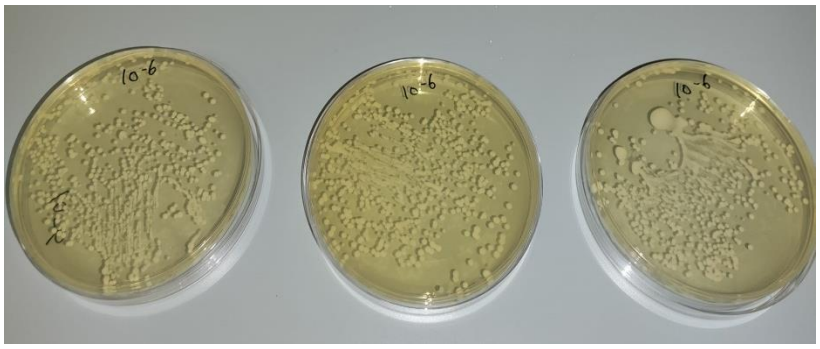

Figure 2: Colonies grown from the streaking of a serially diluted active dry yeast sample of 10^{-6} dilution, streaked on three malt extract agar plates. The colonies were once again considered too numerous to count.

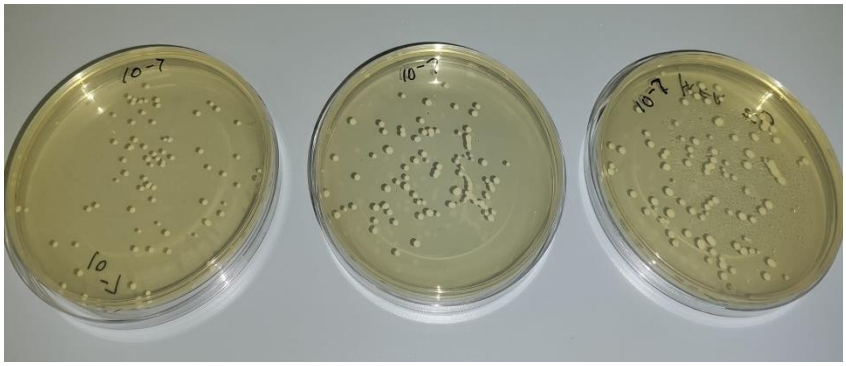

Figure 3: Colonies grown from the streaking of a serially diluted active dry yeast sample of 10^{-7} dilution, streaked on three malt extract agar plates. The colonies were found to lie within the ideal range of 30 to 300 colonies and used for viability analysis of active dry yeast cells.

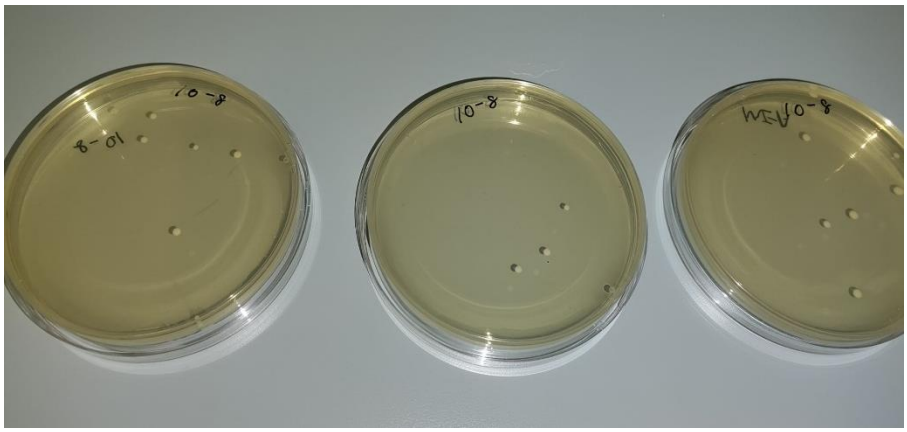

Figure 4: Colonies grown from the streaking of a serially diluted active dry yeast sample of 10^{-8} dilution, streaked on three malt extract agar plates. The colonies were considered too few to count.

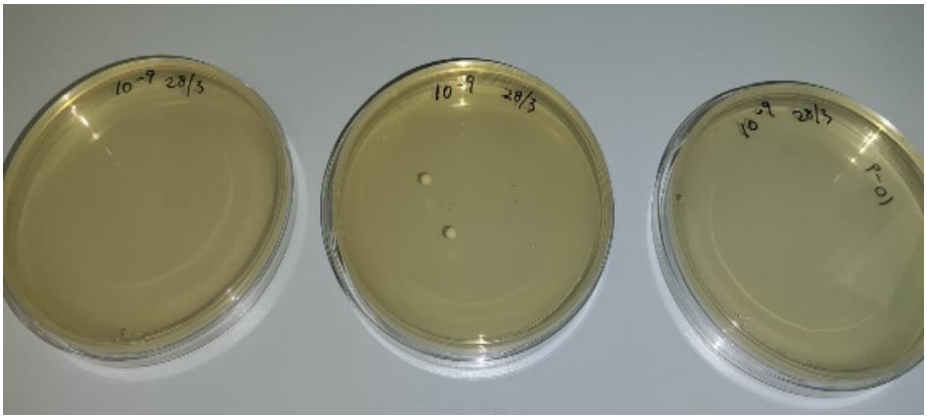

Figure 5: Colonies grown from the streaking of a serially diluted active dry yeast sample of 10^{-9} dilution, streaked on three malt extract agar plates. The colonies were once again considered too few to count.

VII. Results from students t-test

	FCM	Colony Counting	Microscopy
Replicate 1	2.33E+10	1.01E+10	1.34E+10
Replicate 2	2.08E+10	1.01E+10	1.58E+10
Mean	2.21E+10	1.01E+10	1.46E+10
Variance	3.13E+18	0	2.88E+18

Figure 1: Results from students t-test performed to estimate the difference between FCM (variable 1) and colony forming unit (variable 2) where the P(T<=t) two-tail value was found to be 0.0663; and FCM (variable 1) and microscopy analysis (variable 2) where the P(T<=t) two-tail value was found to be 0.2022 respectively, indicating no statistically significant difference in both cases.