Flow cytometry analysis of cell viability of *Lactobacillus reuteri* during growth under different conditions

Master thesis

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Abstract

Lactobacillus reuteri is a probiotic bacterium which when consumed or administered in certain recommended dosages can provide a number of health benefits to the consumer. To have an effect on the consumer, these bacteria must survive until they reach the gut. Process conditions during production of the cells determine their ability to adapt to subsequent environmental shifts during storage and administration. In this project, different conditions during shake flask cultivation were studied to determine the best conditions with respect to growth rate and cell viability as determined by flow cytometry.

Specifically, the effect of growth temperature, agitation and supplementation of citrate and acetate to MRS medium was studied. 44°C gave the highest growth rate and cell titers, as well as a survivability of 96% of the cells during the first 8 hours of fermentation. Agitation of the cells in a shake flask incubator was found to be inhibitory for growth as well as detrimental to the survivability of the cells.

In addition, a mixture of CFDA (carboxy fluorescein diacetate) and PI (propidium iodide) as fluorescent dyes to determine cell viability were benchmarked to SYBR green and PI. It was found that CFDA can be used instead of SYBR green to reach comparable results. The best assay conditions were to use an OD620 of 0.1, 10 mM CFDA, and 1.5 mM PI in a PBS buffer.

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Introduction

Lactobacillus reuteri (*L. reuteri*) is a gram positive, heterofermentative and facultative anaerobic bacterium. It can produce a variety of metabolites, which are known to have antimicrobial properties, such as lactic acid, acetate, ethanol, reuterin and reutericyclin. They are capable of growing in both anaerobic and aerobic environments, albeit some strains show a low tolerance to oxygen and generally prefer acidic conditions. One of the main reasons *L. reuteri* is a commonly studied bacterium is the fact that it exhibits probiotic properties.

L. reuteri most commonly colonize the gastrointestinal tracts of many mammals, including humans. Its robustness in terms of being able to grow in acidic conditions, in a range of different temperatures, and in the presence or absence of oxygen makes it an ideal candidate to survive the harsh conditions of the human body. Its incidence in the microbiota of different individuals tends to vary depending on the age of the host, multiple environmental factors and genetic variation (Scepanovic *et al.,* 2019). Clinical trials have shown that a daily intake of 100 million colony forming units (cfus) is considered as safe and beneficial to the consumer. *L. reuteri* confer a number of probiotic effects to the host which include better digestion, prevention and treatment of certain health conditions such as diarrhoea, alcohol induced liver diseases and Irritable Bowel Syndrome (IBS), lower cholesterol and blood pressure, and a boosted immune system, among other benefits (Goldin, 1998). They have also been shown to produce important nutrients such as the B group vitamins, folate and cobalamin (Rossi *et al.,* 2011;Taranto *et al.,* 2003). There have been no documented major side effects associated with the use of probiotics. Extensive research on the health benefits probiotics have to offer and the spread of this information has led to a demand for highly effective and quality probiotic products.

The choice of the probiotic bacteria to be used in the product is most often made from the bacteria that inhabit the gastrointestinal system of humans. The most commonly used bacteria in probiotic products belong to one of the *Lactobacillus* or *Bifidobacterium* genera, although other genera such as *Escherichia*, *Bacillus* and *Saccharomyces* (a yeast) have been used as the source for the probiotics (Mu *et al.,* 2018).

The production process of probiotics consists of various different steps with different environmental conditions that can affect the survivability and viability of the *L. reuteri* cells from the initial step of the cultivation of the cells to the final step which involves the freeze drying of the harvested cells to generate the final powdered product. This powdered product is then formulated depending on the form of the final product that is being produced (capsules, chewable tablets, etc). Each step can therefore be optimized to maximize the survivability and viability of the cells in the final product.

Flow cytometry (FCM) is a rapid and high-throughput technique for analysing various cell properties of cell cultures at the single-cell level and has high potential to be used as process analytical technology (PAT) to achieve a better understanding and control of cell culture responses to environmental shifts in conditions.

The use of Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA) to determine the viability of bacterial cells using Flow cytometry is becoming increasingly popular. CFDA is a stable, cell-permeable, fluorescein-based dye. It's 2 acetate side chains makes it highly membrane permeable. It is a minimally fluorescent molecule until it diffuses into the cells, where intracellular esterases cleave the acetate groups to formCarboxyfluorescein Succinimidyl Ester (CFSE). CFSE bind covalently to intracellular amino groups via its succinimidyl groups, making it a stable and well retained molecule inside the cells. CFDA is typically combined with Propidium Iodide (PI) to analyse the viability of cells. PI cannot permeate into live cells, and therefore it can be used to detect the presence of dead cells in a population.

The aim of this project was to evaluate different growth conditions of *L.reuteri* with respect to the growth rates and the survivability of the cells, while monitoring the production of different metabolites during the growth. Optimization of the CFDA concentration needed to efficiently stain *L.reuteri* cells for flow cytometric assay to measure the viability of the cells was also done.

Materials and Methods

Strains and medium

The strain used in the experiments was *Lactobacillus reuteri* DSM 17938, provided by Biogaia AB, Stockholm, Sweden. The medium the *L. reuteri* cells were grown on was MRS broth (Merck, USA).

Pre-cultivation

A pre-culture was prepared prior to each cultivation experiment. 100μl of *L.reuteri* cells, which were stored in vials kept at -80°C, were inoculated and incubated in 10ml of sterilized MRS media contained in a 50ml falcon tube, for approximately 16 hours.

Cultivation conditions

For the CFDA-PI assay, 4 falcon tubes, each containing 10ml filtered MRS media, were inoculated with 100μl of live cells. The cells were thawed out prior to the experiment by placing the frozen vials in a water bath at 37°C for 5 minutes. The 4 tubes were then incubated at 37°C. After approximately 4 hours, 2 of the tubes were transferred to 70°C. These 2 tubes served as the dead control. The 4 tubes were then further incubated for an hour. The 4 cultures were then centrifuged for 10 minutes at 10°C with a speed of 3000 rpm. The supernatants were then discarded, and the cell pellets collected at the bottom of the tubes were mixed well with PBS buffer. 50μl of the PBS buffer containing the cells was mixed with 950μl PBS buffer to give a 20X dilution, and an OD620 of approximately 0.1.

L. reuteri was grown in the following conditions: 30°C (without shaking), 37°C (with and without shaking), 44°C (without shaking), and 50°C (without shaking). The cultures were shaken in Erlenmeyer flasks in a shaking incubator at 150rpm. The pre-culture was made as described above. The amount of pre-culture to be added to the flasks was calculated so that the Optical Density (OD) of the inoculated culture was 0.1 at the start of the cultivation. The OD of the pre-culture was first checked using a spectrophotometer (Fisher Scientific, USA) at an absorbance of 620nm and then the following formula was used to calculate the volume of pre-culture to be added: $OD1 \times V1 = 0.1 \times V2$, where OD1 is the OD of the preculture, V1 is the volume of pre-culture to be added and V2 is the volume of media in the flasks. The cultures were grown in Erlenmeyer flasks containing 100ml of sterilized MRS media, in the desired conditions. The MRS media was sterilized by autoclaving the flasks containing the media for 20 minutes at a pressure of 0.5 Bars.

Preparation of CFDA-PI dye mixes

Four different CFDA-PI mixes were made, each having a different CFDA concentration and a fixed PI concentration. The 4 mixes were prepared with the following CFDA concentrations – 10 mM (recommended, as stated by the manufacturer, Sigma-Aldrich, USA), 20 mM (double), 5 mM (half) and 0 mM. Preparation of the dye mixes was started off with the 20mM concentration dye. 0.04 ml CFDA dye was mixed with 1.88 ml distilled water in an Eppendorf tube. 0.96 ml (half the volume of the double concentration mix) was then transferred to another Eppendorf tube, which already contained 0.96ml distilled water, giving a total of 1.92ml. This gave the recommended concentration mix. Once again, 0.96ml of the 10mM concentration mix was mixed with 0.96ml distilled water in an Eppendorf tube which was labelled as the half concentration mix. The zero-concentration mix Eppendorf tube contained just 0.96ml distilled water. Finally, 0.04ml PI dye was mixed into each of the four tubes.

Flow cytometry analysis

To prepare the CFDA assay samples for flow cytometry, 3 tubes, each containing 900μl of PBS buffer were prepared. 100μl of live cells from one of the tubes kept at 37°C was transferred into the first tube. After thorough vortexing, 100μl was pipetted into the second tube from the first tube after which, 100μl was again pipetted into the third tube from the second tube. This gives a 1000X dilution of the live cells in PBS buffer. 200μl of the live cells diluted 1000 times were then transferred into 4 different Eppendorf tubes. 200μl of each of the 4 different dye mixes (20mM CFDA + 1.5 mM PI, 10mM CFDA + 1.5mM PI, 5mM CFDA + 1.5mM PI, 0mM CFDA + 1.5mM PI) prepared before were then mixed into the respective tubes. Similarly, 12 other Eppendorf tubes were prepared, 4 of them containing live cells from the second tube kept at 37°C and 8 tubes containing dead cells from the 2 tubes kept at 70°C. The tubes were then incubated in darkness for 15 minutes before being analysed in the flow cytometer (BD Accuri C6 flow cytometer, USA). To test the SYBR green-PI mix, a 1000X dilution of cells (both live and dead) was prepared. 500μl of the diluted cells was then mixed with 6μl of the CFDA-PI mix and incubated in darkness for 10 minutes at 37°C before being analysed by the flow cytometer.

The flow cytometry data obtained was then analysed using the software FlowJo (version 10.6.1). The files were exported as FCS files from the flow cytometer before analysing them using the FlowJo software. The gating strategies used to isolate the different cell populations can be seen in Appendix III.

HPLC analysis

During the growth of the cells, samples were taken at regular intervals to be analysed using High Performance Liquid Chromatography (HPLC), as described previously (Årsköld *et al.,* 2008). This was done to follow the concentration levels of the various compounds produced or the substrate being consumed during the cultivation. The measured metabolites were citrate, acetate, ethanol and lactate, and the sugar glucose.

Shake flask stress experiments

The *L. reuteri* cells were incubated in a flask containing 100ml of MRS medium at 37°C for 24hours (showing an OD ≈ 8.8). The cultivation was performed without shaking. The cultivation broth was then poured in equal amounts into 2 falcon tubes and centrifuged at 3000rpm at 10°C for 10 minutes. The supernatants were then discarded and 50ml each of PBS buffer and filtered MRS medium was poured into one tube each. The tubes were vortexed until the cells were thoroughly mixed with PBS and MRS. 25ml from each of the tubes was then poured into 4 flasks, all with baffles. 2 of the flasks (1 containing PBS and the other MRS) were sparged with N_2 gas for approximately 30 minutes to flush out all or most of the oxygen in the flasks. The 4 flasks were then shaken at 37°C for 24 hours in a shaking incubator. Samples were taken for Flow cytometry analysis of cell viability at the beginning of the shake incubation (0 hours), 5 hours and 24 hours after the start of the incubation.

Results and Discussion

Effect of temperature and shaking on cell growth

To study the tolerance of *L. reuteri* to elevated temperature, cells were cultivated in MRS medium in incubators set at temperatures between 30-50 degrees (Figure 1). Viability of cells after 8 hours of cultivation were determined with flow cytometry and using the fluorescent dyes SYBR green and PI. The gating strategy to define cells from debris and background noise, and gating of live and dead cells is described in Appendix III.

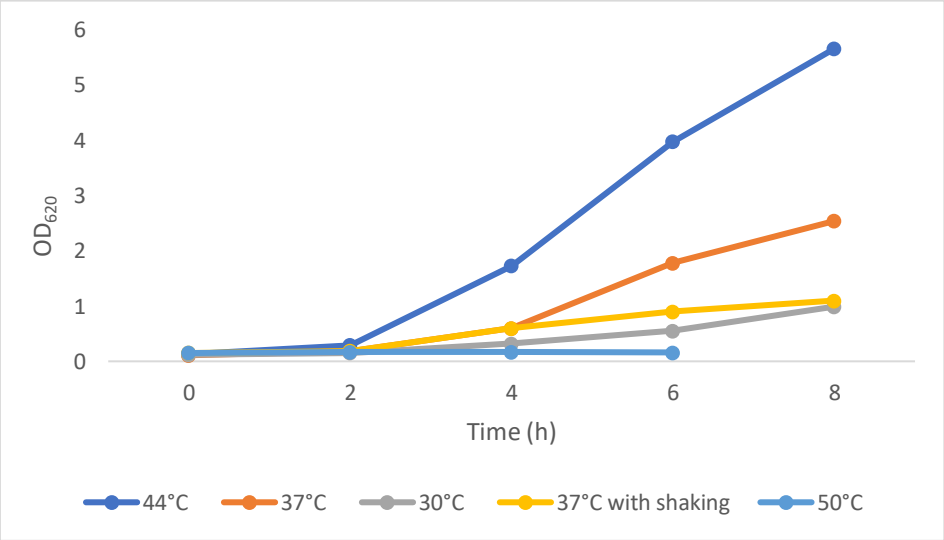


Figure 1.Cell growth in MRS at different temperatures and conditions (with and without agitation)

The highest growth rate and the fastest generation time were seen at a temperature of 44°C (Table 1). The cells grown at 30°C showed a very low growth rate and high generation time. So, 30°C does not seem like a suitable temperature to grow the cells at. The cells were also incubated at 50°C, but they failed to enter the exponential phase of growth (showing no increase in the OD of the culture, measured to be 0.16) even after 6 hours of incubation. The reason for this could be an extended lag phase of the cells at 50°C, or it probably is a too high temperature for the cells to initiate growth altogether.

Cell growth of the cells at 37°C was higher for the cells when not being shaken. This implies that the agitation was detrimental to the growth of the cells and kept the cells in a lag phase for a longer period of time.

From these results, it can be assumed that 44°C is the optimal temperature (or is close to the optimal temperature) for the growth of the *L.reuteri* cells, while agitation slows down the growth of the cells. However, the growth after 8 hours was not measured, and effects caused by prolonged exposure at 44°C may occur.

Flow cytometry analysis was performed to evaluate the different growth conditions with respect to the rate of cell survival. It was performed on cells grown at 44°C and 37°C (with and without shaking). Samples were taken from the culture medium after the cultivation was stopped (approximately 8 hours after inoculation of the cells in the medium). The cells were stained with a SYBR green-PI mix. The analysis was done on duplicates.

Table 1. Growth rates and generation times of cells at different cultivation conditions

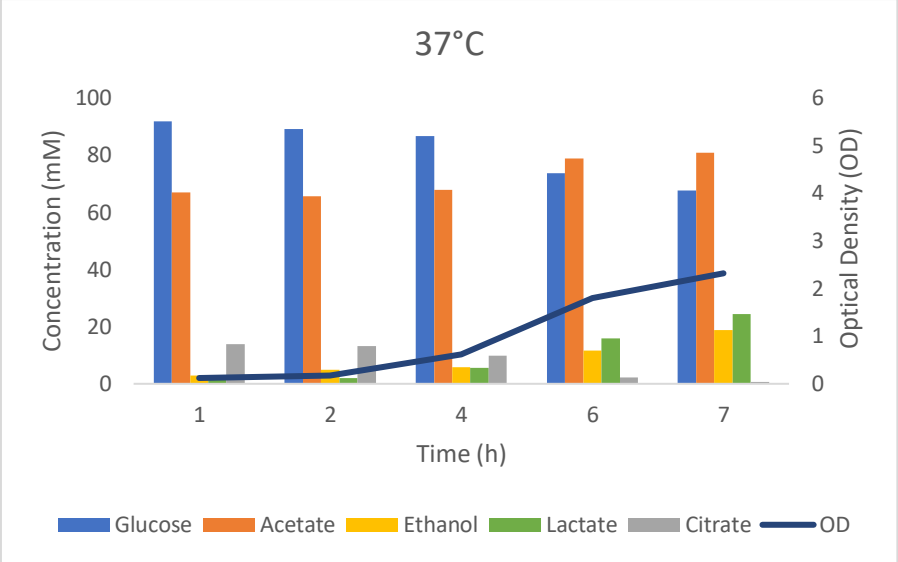
Temperature (°C)	Shaking	OD_{620} (8h)	Growth rate (μ) (h^{-1})	Generation time (T_g) (h)	Cell viability at 8h (%)
44	No	5.7	1.33	0.5	95.7±0.6
37	No	2.5	0.6	1.16	95
37	Yes	1.1	0.2	3.47	85±0.2
30	No	1	0.16	4.33	-

For cells grown at 44°C shows a slightly better cell survivability percentage than cultivation at 37°C.

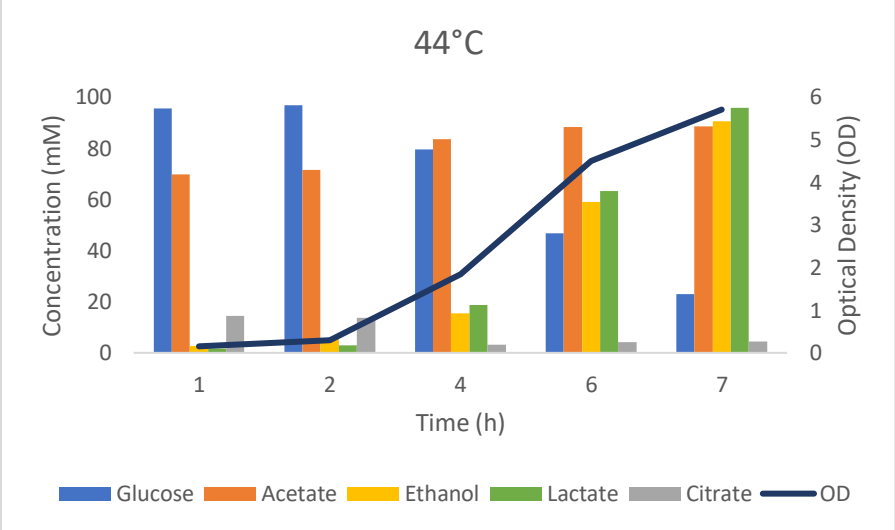
Shaking during cultivation resulted in a survivability rate 10 percent units lower cell viability compared to without shaking. There could be two factors affecting the cells negatively, (i) shear stress and/or (ii) oxidative stress from the increased aeration. The shear stress could have been caused as a result of the cells hitting the inner walls of the cultivation flask while it was being shaken. The oxygen transfer rate also increases because of the agitation of the culture, hence increasing the dissolved oxygen content in the growth medium.

The major compounds produced during the cultivations were acetate, ethanol and lactate (Figures 2a-d). There is a large difference in the amount of glucose present in the culture at the end points of the 4 cultivations [44°C, 37°C (with and without shaking) and 30°C]. This is because the cells grew faster at 44°C and hence consumed more glucose, which acts as the main carbon source. The faster and better growth of cells at 44°C is also the reason why there is a huge difference in the levels of both ethanol and lactate for the 4 cultivation conditions. *L.reuteri* cells use the Phosphoketolase pathway (PKP) to convert glucose into lactate and ethanol (Årsköld *et al.,* 2008). On the other hand, the acetate levels do not differ by much during the cultivations. Acetate produced was calculated to be 0.78g/L or 13 mM (37°C) and 1.08g/L or 18 mM (44°C). Ethanol produced was calculated to be 0.83g/L or 18 mM (37°C) and 4.38g/L or 95 mM (44°C). The acetate produced to ethanol produced ratio (ace_{prod}/et_{prod}) was 0.72 (37°C) and 0.2 (44°C). Citrate, which was present in very small amounts in the initial culture media, was consumed minimally by the growing cells. Citrate could act as an electron acceptor and therefore increase cell growth (Årsköld *et al.,* 2008).

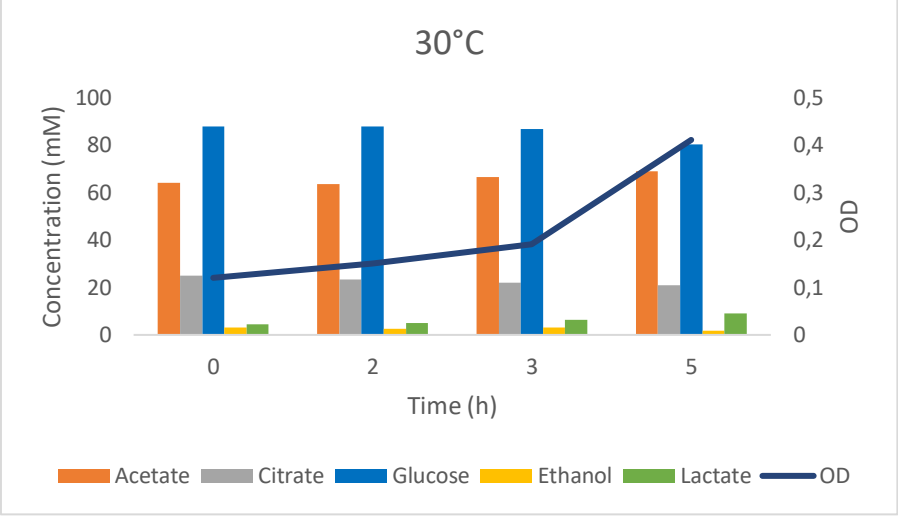
a)


37°C
Concentration (mM)
Optical Density (OD)
Time (h)
Glucose
Acetate
Ethanol
Lactate
Citrate
OD

b)


44°C
Concentration (mM)
Optical Density (OD)
Time (h)
Glucose
Acetate
Ethanol
Lactate
Citrate
OD

c)


30°C
Concentration (mM)
OD
Time (h)
Acetate
Citrate
Glucose
Ethanol
Lactate
OD

d)

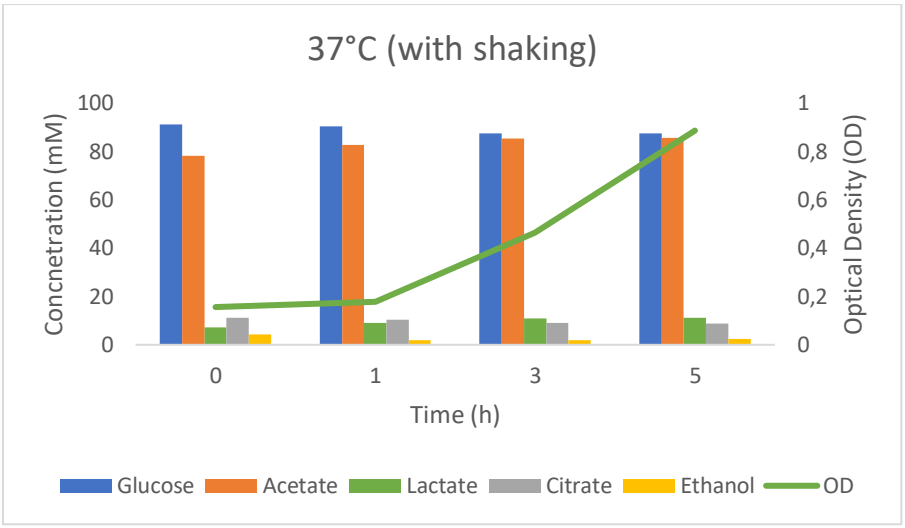


Figure 2. Concentration levels of the different metabolites produced during growth at (a) 37°C, (b) 44°C, (c) 30°C and (d) 37°C (with shaking).

Effect of citrate supplementation on cell growth

To better understand the influence of citrate on the growth of the cells and to determine the concentration-dependence, a set of cultivations in MRS supplemented with citrate was performed. The composition of the MRS media was first checked to find out the amounts of citrate originally present in the MRS media and appropriate amounts of citrate (in the form of di-ammonium hydrogen citrate) were added to the respective culture media to reach the desired concentration levels [5X (10g/L, 52mM) and 10X (20g/L, 104mM) of citrate].

Growth of *L.reuteri* was followed in MRS media supplemented with 5- and 10-times higher citrate concentration. The cultivations were performed at 37°C and samples were taken during the growth for measurement of OD and HPLC analysis and once cultivation was stopped.

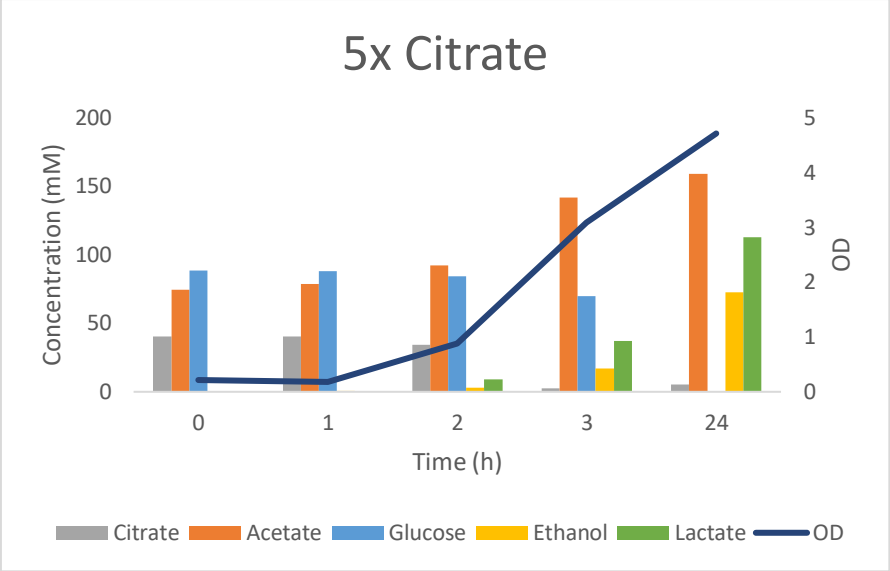


Figure 3. Metabolite levels during growth with 5X citrate over time.

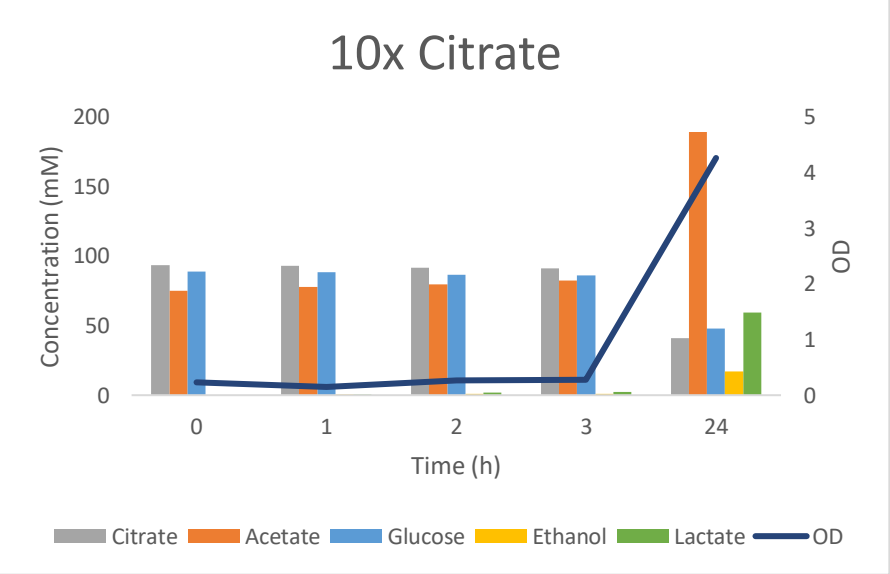


Figure 4. Metabolite levels over time during growth with 10X citrate concentration.

Comparing the growth of cells seen in the 5X (52mM) and 10X (104mM) citrate concentration mediums, growth started faster in the 5X citrate medium (OD levels started increasing after an hour while in 10X citrate medium, increase in OD was seen only after 3 hours). The growth of cells seen in 5X citrate medium is also much faster when compared to the growth in medium at 37°C without any added citrate, whereas the growth of cells is much slower in the 10X citrate medium compared to medium without added citrate, ascertained from the OD levels seen during growth. A slight increase in the acetate level can be seen after 2 hours in the 5X citrate medium. The acetate level seen at the end of 24 hours in the 10X citrate medium is quite higher than what is seen in the 5X citrate medium. The levels of ethanol and lactate in the medium differed quite a lot at the end of 24 hours in both 5X and 10X citrate mediums, which is not the case seen in any of the other cultivations. The acetate produced was

calculated to be 5.04g/L(84mM) (5X) and 6.85g/L(114mM) (10X). The ethanol produced was calculated to be 3.32g/L(72mM) (5X) and 0.74g/L(16mM) (10X). The ace_{prod}/et_{prod} was 1.2 (5X) and 7.1 (10X). These values are much higher (especially for the 10X citrate medium) than those of growth in MRS medium without added citrate. This is because the addition of citrate to the medium causes the conversion of acetyl phosphate into acetate instead of ethanol, which is said to increase the efficiency of the PKP, hence increasing the metabolic activity of the cells (Årsköld *et al.,* 2008). At the end of 24 hours, citrate and more interestingly, glucose was not completely consumed by the cells in the 10X citrate medium. This could mean that presence of citrate in high concentrations could affect the ability of the cells to take up glucose and grow properly. Citrate being left over also means that the growing cells will only consume citrate up to a certain level.

Flow cytometric analysis after 8 hours of cultivation was also done to see the effects of 5X and 10X citrate concentrations on the survivability of the cells.

Table 2. Live and dead cell percentages for 5X and 10X citrate cultivations.

Concentration	Live cells (%)	Live cells average (%)	Dead cells (%)	Dead cells average (%)
Citrate 5X	96	96.1	2.72	2.59
	96.2		2.46	
Citrate 10X	67.9	67.6	28.5	28.8
	67.3		29.1	

The cell viability was much higher when the citrate concentration was 5X when compared to 10X. Presence of citrate at 104 mM will slow down the growth rate of the cells and also adversely affect the viability of the *L.reuteri* cells.

Effect of acetate supplementation on cell growth

Acetic acid is a weak acid and frequently used to inhibit cell growth of many different microorganisms. MRS already contain a relatively large amount of acetic acid, however given that the pKa of acetic acid is 4.8, most of it is in a dissociated form at pH 7, which was the starting pH used in the experiments. The effect of acetate on cell growth and metabolism was investigated by growing the cells in 2- and 4-fold higher starting concentrations of acetate (10g/L (167mM) and 20g/L (333mM) compared to 5g/L in MRS medium).

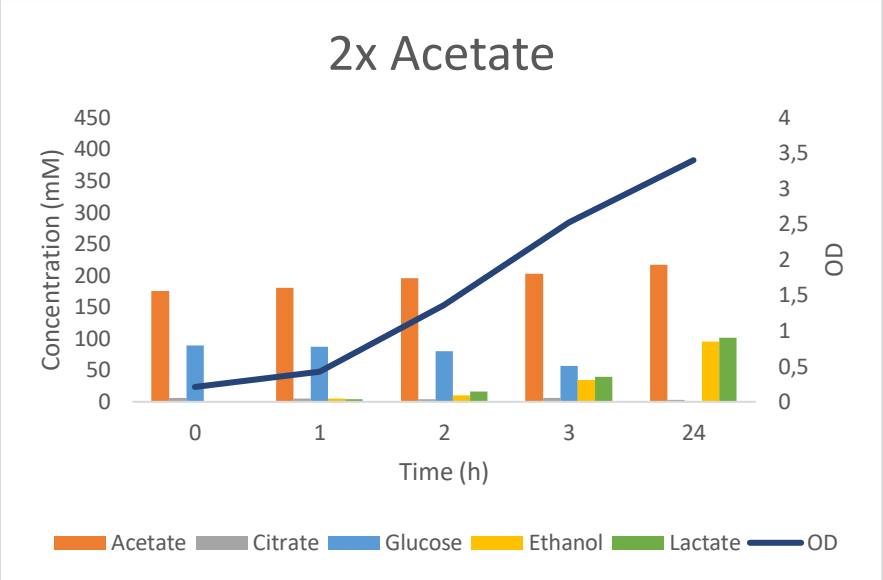


Figure 5. Metabolite levels over time during growth with 2X acetate concentration.

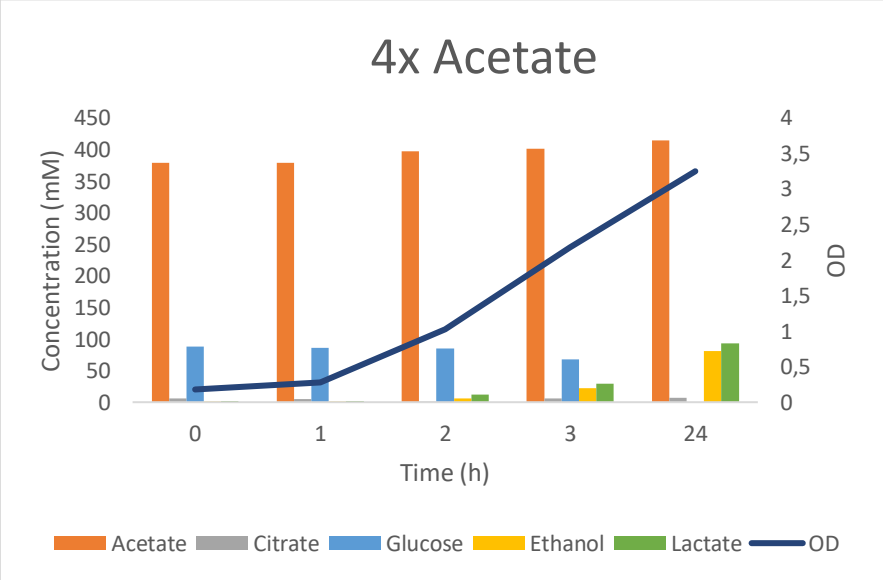


Figure 6. Metabolite levels over time during growth with 4X acetate concentration.

The growth of the cells in 2X and 4X acetate medium is much faster when compared to the growth in medium without added acetate at 37°C (ascertained from the measured OD levels during the growth of the cells). This implies that addition of acetate to the growth medium can help in the growth of *L.reuteri* cells. However, the amount of glucose consumed and the levels of all the metabolites produced during growth in the 10g/L and 20g/L acetate concentration media were similar (Figures 5 and 6).

Flow cytometry analysis of the cell viability at 8 hours showed that addition of acetate to the medium reduced the cell viability (Table 3). Cells grown in the 2X acetate medium showed a lower cell survival rate than the cells grown in 4X acetate medium. Not much can be concluded about the effect of acetate on the survivability of the cells.

Table 3 Live and dead cell percentages for 2X and 4X acetate cultivations.

Concentration	Live cells (%)	Live cells average (%)	Dead cells (%)	Dead cells average (%)
Acetate 2X	74.5	74.3	16.9	16.65
	74.1		16.4	
Acetate 4X	93	93.1	3.70	3.375
	93.2		3.05	

Evaluation of the CFDA-PI assay to determine viability of cells

To determine the optimal CFDA concentration for the measurement of the viability of the *L.reuteri* cells by flow cytometry, the CFDA concentration was varied between 0-20mM while the PI concentration was kept constant at 1.5mM. After the flow cytometry analysis of the samples was concluded, the data obtained was analysed using the FlowJo (version 10.6.1) software. To estimate the viability of the cells, the percentage of live cells stained by CFDA were correlated to the percentage of live cells stained by SYBR green.

The gating methods used to separate the various cell populations can be seen in Appendix III.

Table 4. The percentages of live cells seen for the samples tested with the different dye mixes.

Concentration of dye tested (CFDA + PI)	Live cells (%)
20mm + 1.5mm	65±3
10mm + 1.5mm	97±1
5mm + 1.5mm	98±0.1
0mm + 1.5mm	0

The highest percentage of live cells (98%), which corresponds to the viability of the cells, are shown by the samples tested with the 5mM CFDA concentration mix. The samples tested with the 10mM CFDA concentration mix showed a slightly lower live cell percentage (97%).The cell viability rate seen for the samples stained with the 20mM CFDA concentration mix is much lower (65%) than the recommended and half concentration mixes. This indicates that the 20mM CFDA concentration is not suitable for assay of the *L.reuteri* cells as it causes a relatively high loss of cell viability.

Further analyses were also performed to determine which CFDA concentration (the choice is now between 10mM and 5mM CFDA) was the most suitable for use when assaying the *L.reuteri* cells. The mean fluorescence shown by the CFDA positive cells (live cells) and the frequency of parent statistics for all the samples were found out using FlowJo. Each of the 2 statistics were then plotted against the CFDA concentrations used.

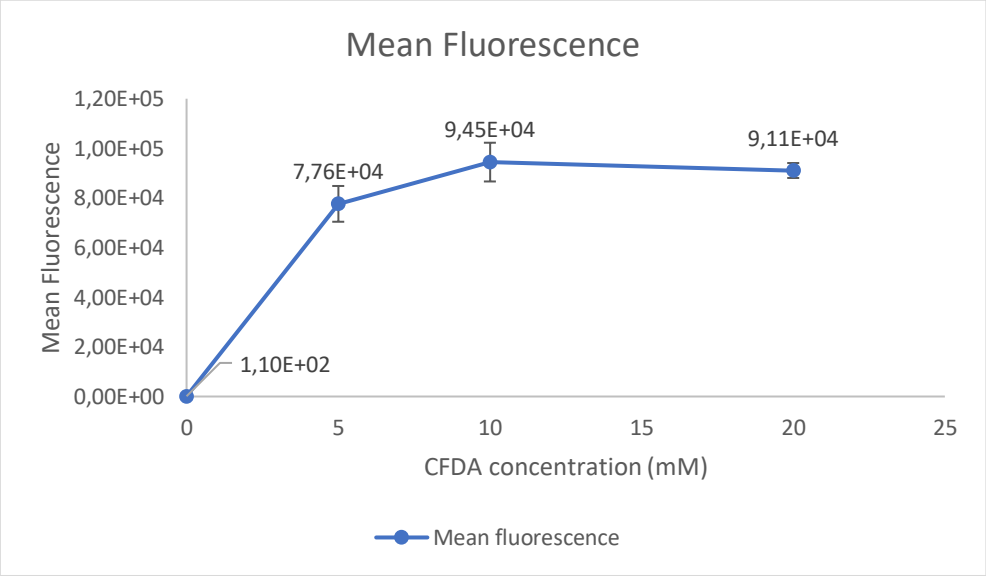


Figure 7. Mean fluorescence of the CFDA positive cells for the different CFDA concentrations.

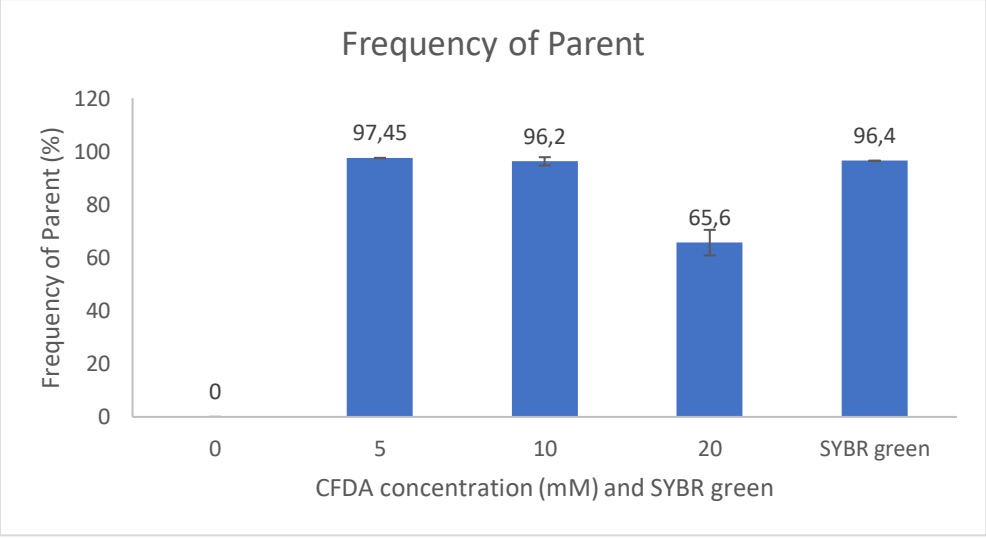


Figure 8. Frequency of parent cells for the different CFDA concentrations and SYBR green.

As can be seen in Figure 6, nothing separates the 10mM and 5mM CFDA concentrations when it comes to the frequency of parent (percentage of live cells in

the total population of the cells), both showing values close to SYBR green (Figure6). According to Figure5, the mean fluorescence exhibited by the live cells gives a much clearer picture as to which CFDA concentration is most suitable. The slope formed when the mean fluorescence was plotted against the CFDA concentrations peaked corresponding to the 10mM CFDA concentration. Saturation is seen as the concentration increases towards 20mM. meanwhile, the 5mM CFDA mix shows a mean fluorescence much lower that the 10mM CFDA mix.

From all the results gathered, 10mM (recommended) seems to be the most suitable concentration of CFDA for the assay of *L.reuteri* cells. The results also point out to the fact that a concentration higher than the optimum can result in a drastic loss of viability of the *L.reuteri* cells.

Shear Stress vs Oxygen

The cells were stained using the optimal CFDA concentration (10mM) determined before and PI dye mix. If both shear stress and oxygen (dissolved in culture broth and in the headspace of the flask) influence the survivability of the cells negatively, the flasks which were not sparged with N_2 gas would show a lower cell survivability rate. On FlowJo, the same gates which were used to first isolate the signal coming from the cells and then to isolate the fluorescence coming from the live and dead cells when 10mM CFDA was tested, was used. The samples were run in the Flow cytometer in duplicates. The following were the live and dead cell percentages seen for the flasks (labelled as PBS N.O, PBS S.S, MRS N.O, MRS S.S):

Table 5. Live and dead cell percentages for sparged flasks containing PBS.

Time (hours)	Sample	Live cells (%)	Average live cells (%)	Dead cells (%)	Average dead cells (%)
0	PBS N.O 1	70.2	70.2	6.4	7.5
	PBS N.O 2	70.2		8.5	
5	PBS N.O 1	0	≈ 79	1.3	2.6
	PBS N.O 2	79		3.8	
24	PBS N.O 1	77.7	80.75	5	3
	PBS N.O 2	83.8		0.9	

Table 6. Live and dead cell percentages for non-sparged flasks containing PBS.

Time (hours)	Sample	Live cells (%)	Average live cells (%)	Dead cells (%)	Average dead cells (%)
0	PBS S.S 1	76.1	73.35	4.7	5.9
	PBS S.S 2	70.6		7	
5	PBS S.S 1	78.2	75.1	3.7	5.6
	PBS S.S 2	72		7.5	
24	PBS S.S 1	84.1	83.1	2.6	2.6
	PBS S.S 2	82.1		2.6	

Table 7. Live and dead cell percentages for sparged flasks containing MRS.

Time (hours)	Sample	Live cells (%)	Average live cells (%)	Dead cells (%)	Average dead cells (%)
0	MRS N.O 1	78.2	78.5	3.2	2.8
	MRS N.O 2	78.8		2.4	
5	MRS N.O 1	80.4	76.9	1.7	3.9
	MRS N.O 2	73.4		6	
24	MRS N.O 1	83.9	84.8	1.1	0.9
	MRS N.O 2	85.7		0.7	

Table 8. Live and dead cell percentages for non-sparged flasks containing MRS.

Time (hours)	Sample	Live cells (%)	Average live cells (%)	Dead cells (%)	Average dead cells (%)
0	MRS S.S 1	82.6	82.7	1.2	1.1
	MRS S.S 2	82.7		0.9	
5	MRS S.S 1	78	78.7	3.6	3.4
	MRS S.S 2	79.3		3.2	
24	MRS S.S 1	81	80.7	3.5	4
	MRS S.S 2	80.4		4.5	

The flasks which were not sparged showed a greater live cell percentage than the flasks which were sparged with N_2, except for PBS N.O (5h) and MRS S.S (24h). The PBS N.O value was probably skewed from the true value because of a mistake made while preparing the sample for Flow cytometry, which gave a reading of 0% live cells. This implies that oxygen could have a positive impact on the survivability of the cells. The cells resuspended in MRS showed higher cell survivability rates than the c ells resuspended in PBS, except for PBS N.O (5h) and MRS S.S (24h) once again.

The average live and dead cells do not add up to a number close to 100 for all the cases. This means that the live and dead cell gates used did not include fluorescence seen from all the cells. Proper CFDA gating protocols need to be established before further analysis can be continued.

Conclusion

In conclusion, *L.reuteri* shows better growth rate and higher cell survivability when grown at 44°C when compared to any other temperature. Agitation of the *L.reuteri* cells during their growth slows down the growth rate and also decreases the viability of the cells. This can be either due to shear stress or increase in the dissolved oxygen concentration in the growth medium, or a combination of both. Further tests showed that oxygen might have a positive influence on the survivability of the cells. However further tests need to be done to verify the effect of oxygen on the survivability of the cells, once an accurate CFDA gating protocol is established.

Addition of a certain amount of citrate (up to 5 times as much as the normal citrate concentration) to the MRS growth medium can increase the growth rate and survivability of *L.reuteri* cells. Increasing the acetate concentration in the MRS media also increases the growth rate of the *L.reuteri* cells. It however does not seem to have any defined effects on the viability of the cells under the investigated conditions.

Finally, a CFDA concentration of 10mM along with a PI concentration of 1.5 mM was determined to be the most suitable concentration for the flow cytometric assay of *L.reuteri* cells.

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Appendices

Appendix I. MRS growth medium components

Appendix II. Calculation of growth rates.

Appendix III. Gating strategies used to analyse Flow cytometry data.

Appendix I. MRS growth medium components.

The following are the contents of the MRS media used for the cultivations along with their concentrations :

Peptone from casein :10g/L

Meat extract : 8g/L

Yeast extract : 4g/L

D(+) glucose : 20g/L

di Potassium hydrogen phosphate : 2g/L

Tween80 : 1g/L

di Ammonium hydrogen citrate : 2g/L

Sodium acetate : 5g/L

Magnesium sulphate : 0.2g/L

Manganese sulphate : 0.04g/L

Appendix II. Calculation of growth rates

All the cultivation experiments were either performed in duplicates or triplicates, so the OD values used for calculations were averages. The growth rate of the *L.reuteri* cells during cultivation under the different conditions can be calculated using the natural logarithm of measured OD values. The natural logarithm of the measured OD values was plotted as a function of the time to generate a growth curve. The linear part of the curve (which usually corresponds with the exponential growth phase of the cells) was then located. The slope of that curve (the x coordinate of the equation of the slope, y = mx+c) gives the growth rate (μ) of the cells during the cultivation. The generation time, which is the time required by the cells in the culture to divide, can also be calculated from the growth rate using the formula, $T_g = \ln 2 / \mu$.

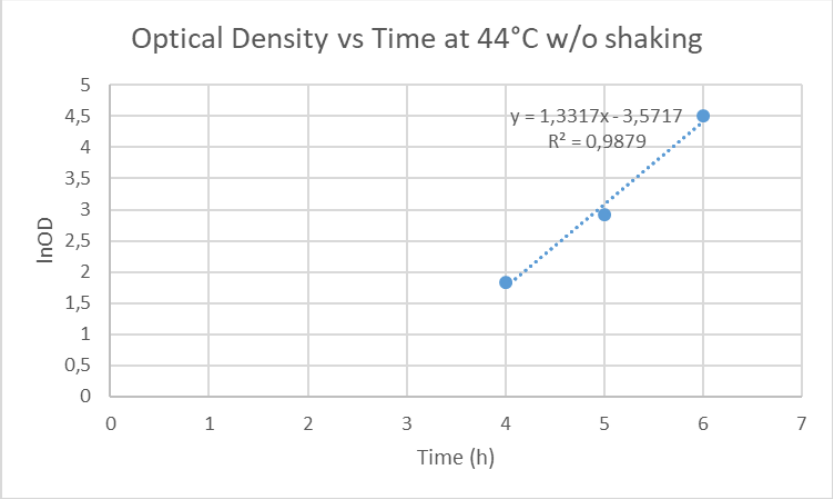

Figure 9. Optical Density vs Time plot for the calculation of the growth rate, μ.

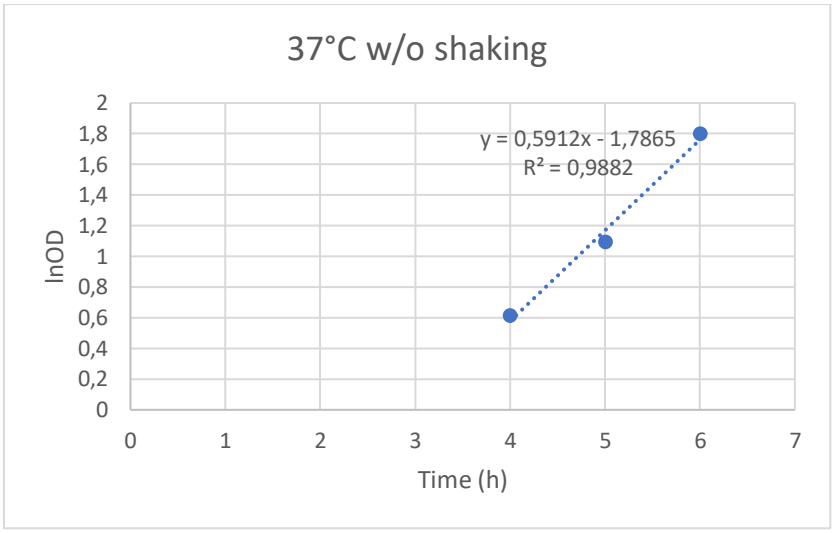

Figure 10.Optical Density vs Time plot for the calculation of the growth rate, μ at 37C.

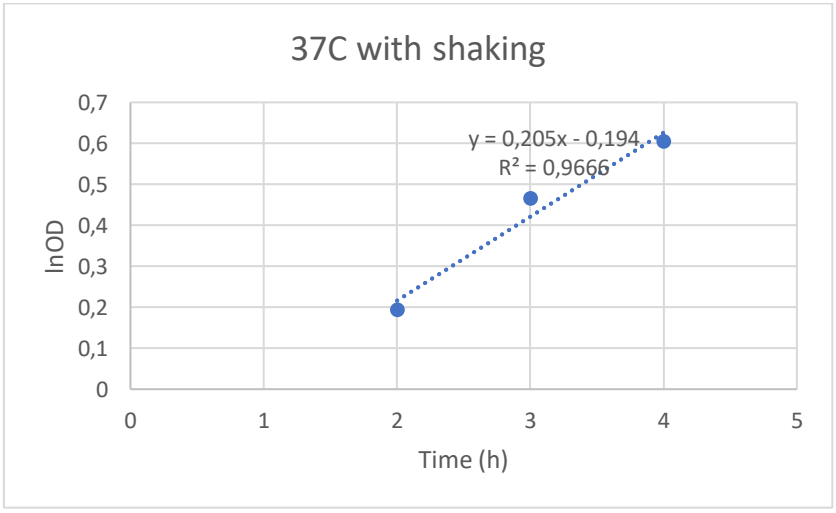

Figure 11. Optical Density vs Time plot for the calculation of the growth rate, μ at 37C with shaking

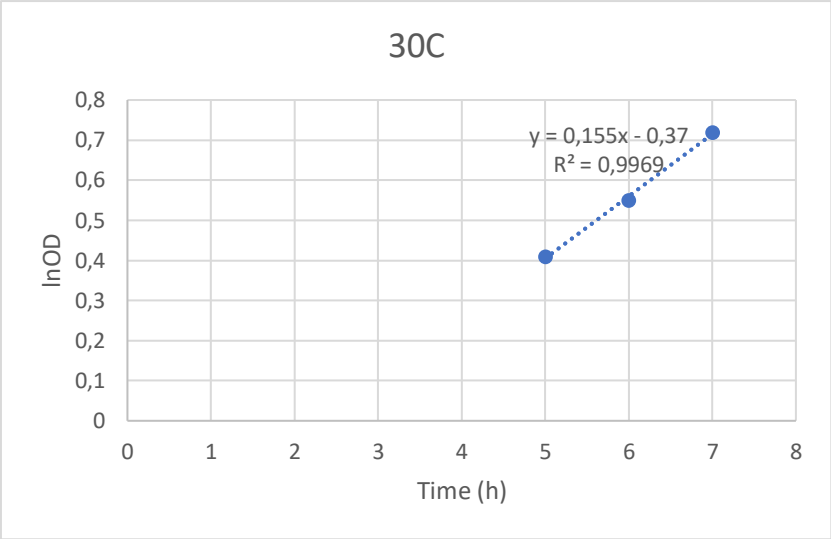

Figure 12. Optical Density vs Time plot for the calculation of the growth rate, μ at 30C

Appendix III. Gating strategies used to analyse Flow cytometry data

The first step of the analysis was to separate the signal received from the specific staining of the cells from the non-specific and background signals (which can come from any debris present in the sample). This was done by visualizing the fluorescence with the forward scattering (FSC) on the x-axis and the side scattering (SSC) on the y-axis, as can be seen in figure 1. The forward scatter gives the relative size of the cell while the side scatter gives the internal complexity or granularity of the cell.

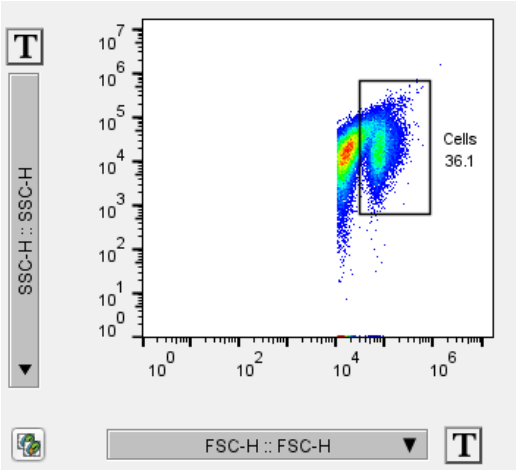

Figure 13. Isolating specific signal from background noise.

After gating the cells based on FSC and SSC, the fluorescent signals were visualised with the FL1 parameter (CFDA, or SYBR-green) on the X-axis and the FL3 parameter (PI) on the Y-axis (Figures 14 and 15). The live cell subpopulation (stained by CFDA and SYBR-green) and the dead cell subpopulation (stained by PI) were gated. The fluorescence seen from the dead control samples was used as a reference while gating the live and dead cell populations. The signals seen from the samples stained with SYBR green-PI mix were gated as coming from live, damaged and dead cells.

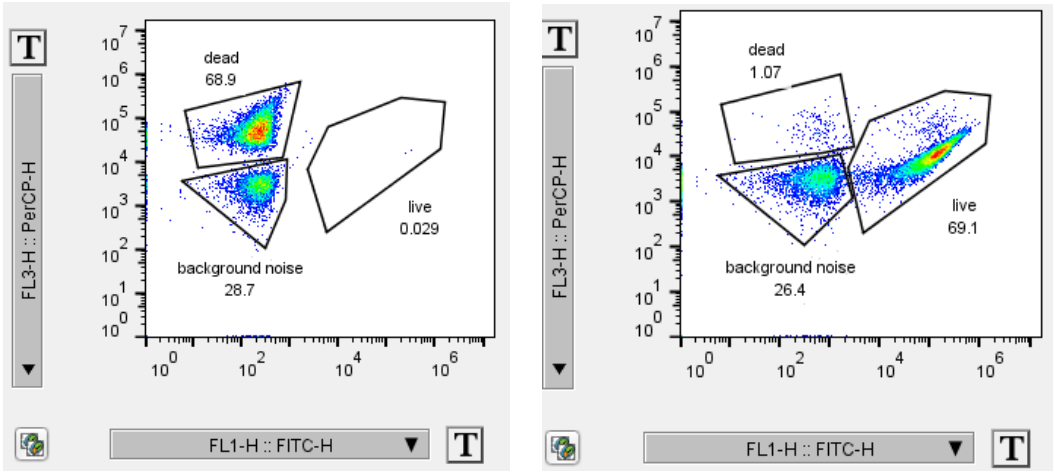

Figure 14. The picture on the left shows the fluorescence from the dead control cells and the picture on the right shows the fluorescence from the live control cells, both stained with the 20mM CFDA concentration mix.

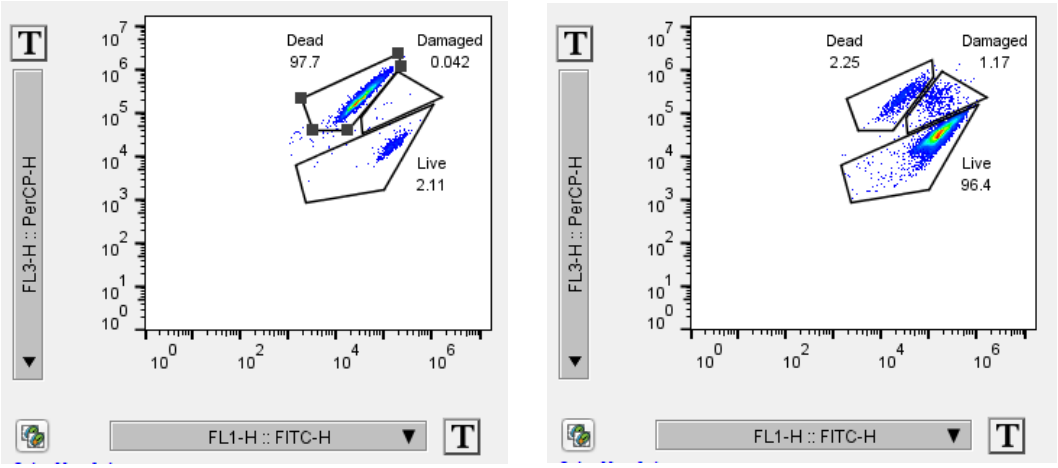

Figure 15. The picture on the left shows the fluorescence from the dead control cells and the picture on the right shows the fluorescence from the live control cells, both stained with the SYBR green-PI mix.

Using this method, the various populations of cells were isolated for all the samples analysed using Flow cytometry.