# Determine the effect of inositol supplementation on fermentation parameters of *Yarrowia lipolytica* in comparison to *Saccharomyces cerevisiae*

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## INTRODUCTION

*Yarrowia lipolytica* and *Saccharomyces cerevisiae* are both important microorganisms for the industrial biotechnology sector. Thus, understanding the consumption, production, and accumulation of certain carbohydrates and metabolites in yeasts are important for exploiting their respective metabolic network for biotechnology applications such as fuels and bioproducts. *Y. lipolytica* is of particular interest because it is an oleaginous yeast that has the ability to naturally accumulate high levels of stored lipid that can represent 25% of its dry mass [6]. *Y. lipolytica* is also the only oleaginous microorganism with developed genetic engineering tools and complete genome sequence that can be used to modify the strain for higher lipid content [6].

Lipid content, especially phosphatidylinositol (PI), of *S. cerevisiae* is influenced by the amount of inositol in the medium [12,13]. Thus, part of our goal is to understand the influences of this phospholipid precursor in all yeast. The *S. cerevisiae* lipid metabolic network is well studied with regards to inositol, but not *Y. lipolytica.* Learning the effect of inositol on neutral lipid, in particular triacylglycerol (TAG), production by *Y. lipolytic*a is important to the biodiesel industry. TAG is of interest because *Y. lipolytica* stores most of its lipid in form of TAG [3]. With a glycerol group attached to three fatty acid chains (palmitic acid, oleic acid, and alpha-linolenic acid), TAG is rich in the fatty acid reserve making it an excellent bioproduct for biodiesel synthesis [9].

Inositol may also have an effect on lipid storage according to proteomic analysis. Morin et al. identified two genes related to lipid storage that were significantly expressed in the late stage of lipid accumulation [4,14]. One of the two genes was SAC1, which encoded for an inositol/phosphatidylinositol phosphatase. This phosphatase is shown to be a key component of *Y. lipolytica*'s lipid body and was involved in the hydrolysis of phosphate from phosphatidylinositol 4-phosphate [14,21]. Knowing that proteins on the lipid body are essential for maintaining the dynamic synthesis and turnover of TAG and SE in the lipid body, the increased activity of Sac1p during the lipid accumulation phase may indicate a connection between inositol and lipid synthesis, though the exact metabolic influence of SAC1 is still unknown.

The main objective of this study is to explore the lipid metabolism of *Y. lipolytica* in response to inositol compared to *S. cerevisiae* under fermentation. Another goal is to study several key parameters such as specific growth rate and metabolite production of *S. cerevisiae* and *Y. lipolytica* under media condition with and without inositol. The study of these parameters is essential for a basic understanding of cellular responses in a lab bench fermenter and in application to scale up fermentation production if desired.

## METHOD

### Micro-organism

Wild type *Saccharomyces cerevisiae* BY 4742 and wild type *Yarrowia lipolytica* ATCC 20460 were used in this study. *S. cerevisiae* BY 4742 strain was obtained from Dr. Susan Henry's

lab. The strain was maintained at -80°C in 80% glycerol stock. *Y. lipolytica* ATCC 20460 was obtained from ATCC. The strain was maintained at -80°C in 20% glycerol stock.

**Cultured condition**

Two types of media were used for *S. cerevisiae* and *Y. lipolytica* cultivation. I+ medium contained 75 μmol of myo-inositol along with other components that can be found in Appendix I. I- medium contained the same components as I+ with the exception of myo-inositol.

**Preparation of inoculum**

*S. cerevisiae* stock stored at -80°C was plated onto I+ and I- plates and incubated at 30°C for 2.5 days (Innova 44 Incubator shaker Series; New Brunswick Scientific Company, Inc.; USA). Five colonies (2 mm diameter) from the I+ plate and 5 colonies (2 mm) from the I- plate were harvested with a sterile loop and suspended in a glass media bottle containing 50 mL of I+ medium and another containing 50 mL of I- medium, respectively. These pre-inoculums were placed in an incubator-shaker (Innova 3100 Water Bath Shaker; New Brunswick Scientific Company, Inc.; USA) at 30°C and 200 rpm for 22 hr. Five milliliters of I+ and I- pre-inoculums were then transferred to glass media bottles containing 250 mL of I+ and I- media under a sterilized hood, respectively. Inoculums were grown in incubator-shaker for 16 hr at 30°C and 200 rpm until inoculating the fermenters.

The preparation for the *Y. lipolytica* culture was the same as *S. cerevisiae* except for the temperature that was set at 25°C. The temperatures were selected as the optimal growth temperature for the respective yeast.

**Batch cultivation**

Optical density (OD) of I+ and I- inoculums were measured using a spectrophotometer (Eppendorf BioPhotometer plus; Eppendorf AG; Germany) at 600 nm before transferring inoculums into 2.5 L BioFLow&CelliGen 310 Fermentor/Bioreactors (Eppendorf AG; Germany) containing 1500 mL of respective I+ and I- media for all experiments except for experiment 3, starting with 1000 mL of I+ and I-. According to the measured OD, adequate amount of inoculums were transferred into the fermenters to start each fermenter with initial OD 0.1. Five mL of antifoam were also added for each fermenter at the start of fermentation. *S. cerevisiae* fermenters were operated at 30°C and 200 rpm for 72 hr. *Y. lipolytica* fermenters were operated at 25°C and 200 rpm for 72 hr. Four experiments were performed with different aeration and pH control listed below. For pH control, 36.5 - 38% Hydrochloric Acid (VWR Cat#BDH3028-2.5LG) and 5N Sodium Hydroxide (J.T Baker Cas#1310-73-2) were used.

Experiment 1: *S. cerevisiae* grew on I+ and I- media under low aeration at 1 standard liter per minute (SLPM).

Experiment 2: *Y. lipolytica* grew on I+ and I- media under low aeration at 1 SLPM

Experiment 3: *Y. lipolytica* grew on I+ and I- media under high aeration at 8 SLPM

Experiment 4: *Y. lipolytica* grew on I+ and I- media under high aeration at 10 SLPM with pH control between 4.5 - 5.5.

Three replicates were performed for I+ and I- for each experiment. I+ inoculum was used to inoculate fermenter 1, 2, and 3 and I- inoculum was used to inoculate fermenter 4, 5, and 6. Experiment 1 was served as a control for lipid analysis since inositol is known to have an effect for *S. cerevisiae* lipid composition.

**Analytical methods**

**OD measurement**

The OD of both *S. cerevisiae* and *Y. lipolytica* were measured using a spectrophotometer at 600 nm throughout the fermentation run. Three OD readings were taken for an average OD for each time point.

**Cell mass quantification**

Cell mass was determined gravimetrically. Fermentation samples were collected throughout 72 hr from each fermenter and stored in 4°C until all the samples were ready for cell mass determination. Fifteen mL of fermentation samples were filtered through 0.4 µm, 47 mm Whatman Nuclepore Track-Etched Membrane (Sigma-Aldrich; USA), and then dried in oven at 105°C for 1 day. Cell mass measurements were made 1 day after filtration.

**Organic acids and carbohydrates quantification**

Organic acids and sugars were determined by HPLC Aminex HPX-87H Organic Acid Column (Bio-Rad, USA) with UV detector at UV 210 nm and RI detector. Samples were eluted with 5 mM-$H_2SO_4$ at 65°C with isocratic flow rate of 0.6 $cm^3$/min and 20 µL injection volume. Concentrations of glucose, glycerol, ethanol, and acetic acid were measured for the *S. cerevisiae* fermentation. Concentrations of glucose and citric acid were measured for the *Y. lipolytica* fermentations.

**Neutral lipids quantification**

Thin layer chromatography (TLC) was used to determine neutral lipid content of *S. cerevisiae* and *Y. lipolytica*. Fermentation samples for each fermenter of *S. cerevisiae* and *Y. lipolytica* were collected at the end of 72 hr, which was when the cells presumably entered deep stationary phase with considerable lipid accumulation. Right after samples were collected, samples were centrifuged at 8,000 rpm for 20 min using a Sorvall Evolution RC Centrifuge with a Fiberlite F8-6 x 1000y Rotor (Thermo Scientific, USA). The cell pellet were resuspended in 25 mL $dH_2O$, and then transferred to 50 mL falcon tubes. The falcon tubes were centrifuged at 6,000 rpm for 10 min using a Fiberlite F13-14 x 50cy Rotor (Thermo Scientific, USA) and the supernatants were discarded. For TLC measurements, 5 mL of 5% TCA was added to cell pellets, vortexed, and incubated on ice for 15 min. Samples were centrifuged at 6,000 rpm for 5 min and washed with 7 mL $dH_2O$. Following the Folch's method, 8 mL of 2:1 chloroform-methanol (v/v) extraction of neutral lipid was used, in addition to adding 1200 µL of 1.8% NaCl [10]. The samples were dried under nitrogen gas and total dry lipid weights were recorded.

Eight-hundred micrograms of total dry lipid weight for each *S. cerevisiae* sample and 400 µg for each *Y. lipolytica* sample were spotted onto silica gel on TLC plate (Sigma-Aldrich, USA). Solvent system of hexane: ethyl ether anhydrous: formic acid (80:20:2 v/v) was used to run the neutral lipids on the TLC plate. The plates were dipped into charring solution consisted of 9.4 mL 85% phosphoric acid and 10 g of cupric sulfate pentahydrate that was added up to 100 mL with water. TLC plates were transferred to a pre-heated 180°C oven for 15 min for the charring of neutral lipids. Plate images were captured by ENDURO GDS touch (labnet international, Inc; Edison, New Jersey). Quantification of neutral lipid was performed with ImageJ program (Image processing and analysis in Java) obtained from NIH.

**Calculation of fermentation parameters**

Specific growth rate [$h^{-1}$], µ, was estimated from experimental data using MATLAB's Statistics Toolbox. Growth curve fit on MATLAB was done by an exponential fit of the following equation to experimental data:

$$f(t) = x_0 \exp(\mu t) \quad (1)$$

The equation was fitted to the data points that were linear on the logarithmic OD graph versus time. The linear region on the logarithmic OD graph was from 1 hr to 11.75 hr for experiment 1, 0 hr to 11.5 hr for experiment 2, and 0 hr to 12 hr for experiment 3.

*S. cervisiae* product yield was calculated for ethanol, acetic acid, and glycerol using the following equation:

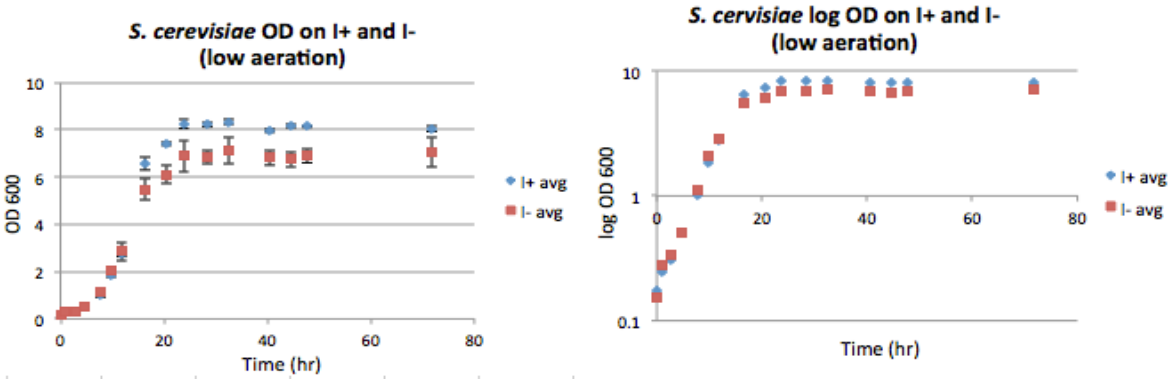
$$\text{Yield [g/g]: } Y = P/S \quad (2)$$

where P was the total amount of product of interest (g/L) and S was the total amount of substrate consumed (g/L) in the fermentation culture

**RESULTS AND DISCUSSION**

**Experiment 1: *S. cerevisiae* on I+ I- (aerated 1 SLPM)**

OD was measured over the course of 72 hr, and temporal plots of OD yielded a sigmoidal growth curve as shown in Figure 1. Error bars were also plotted for each measurement as standard error at each time point for I+ and I-. Results from fermenter 6 (I-) were disregarded as growth was irregular possibly due to contaminated reactor. At steady state, the average OD for I+ was 7.899 and the average OD for I- was 6.657 with standard errors of 0.0678 for I+ and 0.4377 for I-. The cell growth for *S. cerevisiae* on I+ and I- media was significantly different at steady state as no overlapping of error bars occurred in Figure 1a. There was no trend of biphasic growth as *S. cerevisiae* switched from glucose to ethanol consumption since ethanol consumption was possible under aeration. This indicated that cells immediately transitioned into ethanol consumption after glucose was depleted. As a result, there was no evidence of 2 exponential phase separated by a plateau phase.




(a) (b)

Figure 1. *S. cerevisiae* on I+ and I- media under low aeration: (a) OD vs. time graph (b) logarithmic OD vs. time graph.

Specific growth rates for *S. cerevisiae* were summarized in Table 1. I+ and I- had similar specific growth rates with no substantial differences as observed from Table 1 and Figure 1a visually. I+ had an average specific growth rate of 0.2394 $h^{-1}$ and I- had an average specific growth rate of 0.2272 $h^{-1}$. Krause et al. had a growth rate of 0.102 $h^{-1}$ for *S. cerevisiae* on I+, 12% glucose, no aeration and 0.100 $h^{-1}$ on I-, 12% glucose, no aeration, which were lower than the average specific growth rates achieved in this study [13]. The lower specific growth rates could be a result of limited aeration.

Table 1. Specific growth rates and standard errors for *S. cerevisiae* on I+ and I- media under low aeration.

| *S. cerevisiae* (low aeration) | I+ avg | I- avg |
|---|---|---|
| $\mu$ ($h^{-1}$) | 0.2394 | 0.2272 |
| Standard Error | 0.00619 | 0.00545 |

Temporal cell mass concentrations are plotted against corresponding OD measurements in Figure 2 for *S. cerevisiae.* The cell mass dried loosely to the filter paper and some cell mass may had been lost while measuring. The $R^2$ values for the liner fit were 0.956 and 0.839 for I+ and I-, respectively. From this analysis, it seemed that the OD is not sensitive to changes in cell dry mass over ~1.6 mg/mL for I+ and ~1.9 mg/mL for I-. A linear relationship existed between cell mass and OD for OD values below ~7. A linear relationship only for data points below this threshold should be used for future analysis.

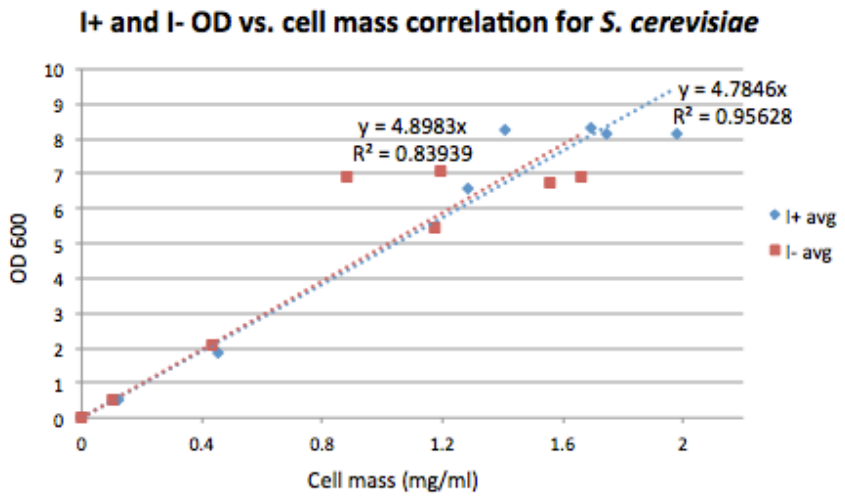



Figure 2. Cell mass correlation with OD for *S. cerevisiae* on I+ and I- under low aeration.

Ethanol catabolism is possible for *S. cerevisiae* under aeration. According to Figure 3, after glucose was depleted at 28.5 hr, *S. cerevisiae* switched from ethanol fermentation to ethanol consumption to continue cell growth. The ethanol concentration reached the highest of 6.68 g/L at 28.5 hr for I+ as compared to 6.40 g/L for I-. The temporal change in ethanol concentration follow similar trends between I+ and I-. Even though more acetic acid was produced for I- from 20 hr to 48 hr, acetic acid concentration reached the same level for I+ and I- at 72 hr. Glycerol concentration reached a steady state level for both I+ and I- after 28.5 hr with I+ having 0.1 g/L more glycerol than I- at steady state. Glycerol concentration remained the same after *S. cerevisiae* switched from consuming glucose to ethanol, yet acetic acid concentration continued to increase. The activate metabolic pathways were different for cells growing under different sugar sources, demonstrating that growing microorganism on different sugar source may greatly affect the end products.

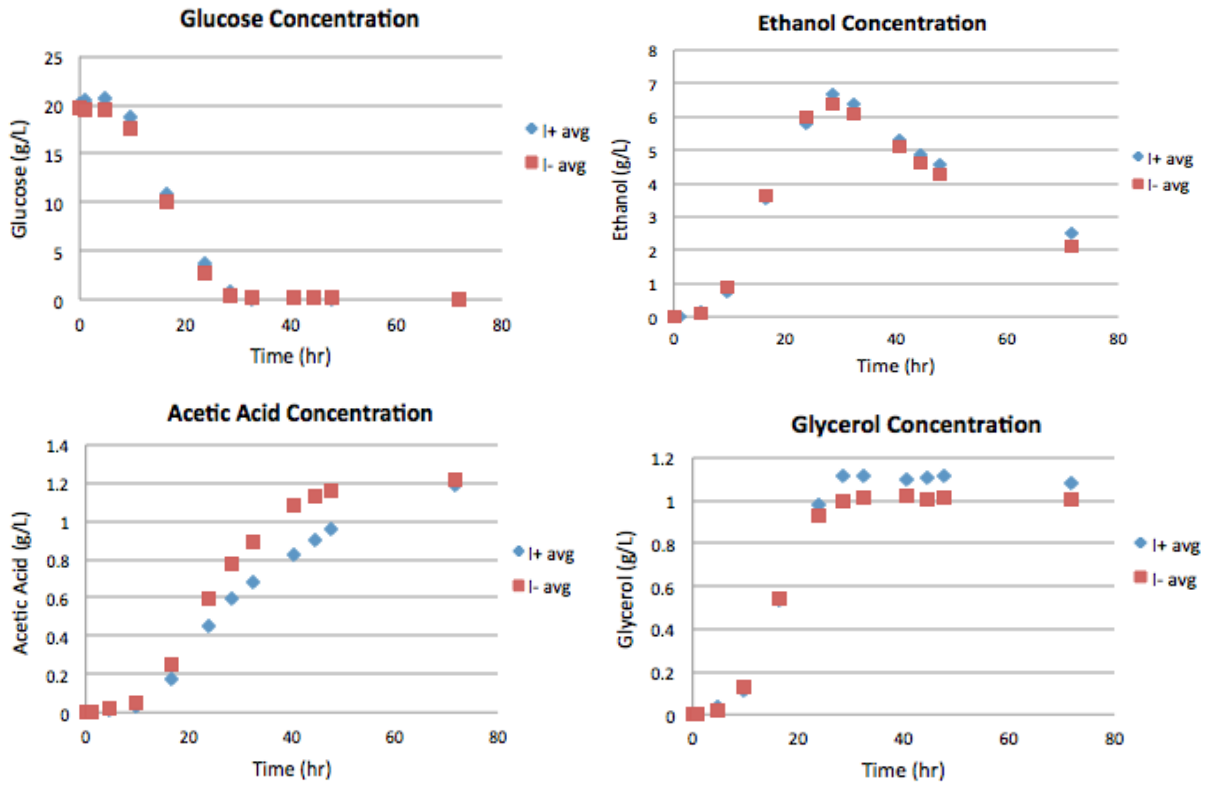



Figure 3. Glucose, ethanol, acetic acid, and glycerol concentrations of *S. cereivisiae* fermentation on I+ and I- under low aeration.

Inositol's influence on fermentation performance of *S. cerevisiae* can be further analyzed through metabolite yields. As seen in Figure 4, I+ had a higher yield for ethanol and glycerol than I-. I+ produced a higher maximum ethanol yield of 0.57 g/g glucose at 9.75 hr than I-, which produced a maximum ethanol yield of 0.42 g/g glucose. *S. cerevisiae* supplemented with inositol contained higher concentration of PI in the cell membrane, allowing the cells to have higher tolerance for ethanol [13]. Higher cell viability in I+ medium in turn allowed for higher ethanol production. I+ also produced a higher maximum glycerol yield of 0.084 g/g glucose at 9.75 hr in comparison to I- maximum glycerol yield of 0.060 g/g glucose. Glycerol yield leveled off for both I+ and I- after entering steady state at 25 hr. As for acetic acid, I- had a higher yield throughout 72 hr as

compared to I+. The maximum acetic acid yield of 0.054 g/g total carbon occurred at 44.5 hr for I- and the maximum acetic acid yield of 0.050 g/g total carbon occurred at 72 hr for I+. The total carbon unit here included the amount of glucose plus ethanol consumed up to the time point. For both cases, the maximum yield both occurred when the cells were grown on ethanol. This may suggest that growing *S. cerevisiae* on ethanol would drive the metabolic flux toward acetic acid production pathway.

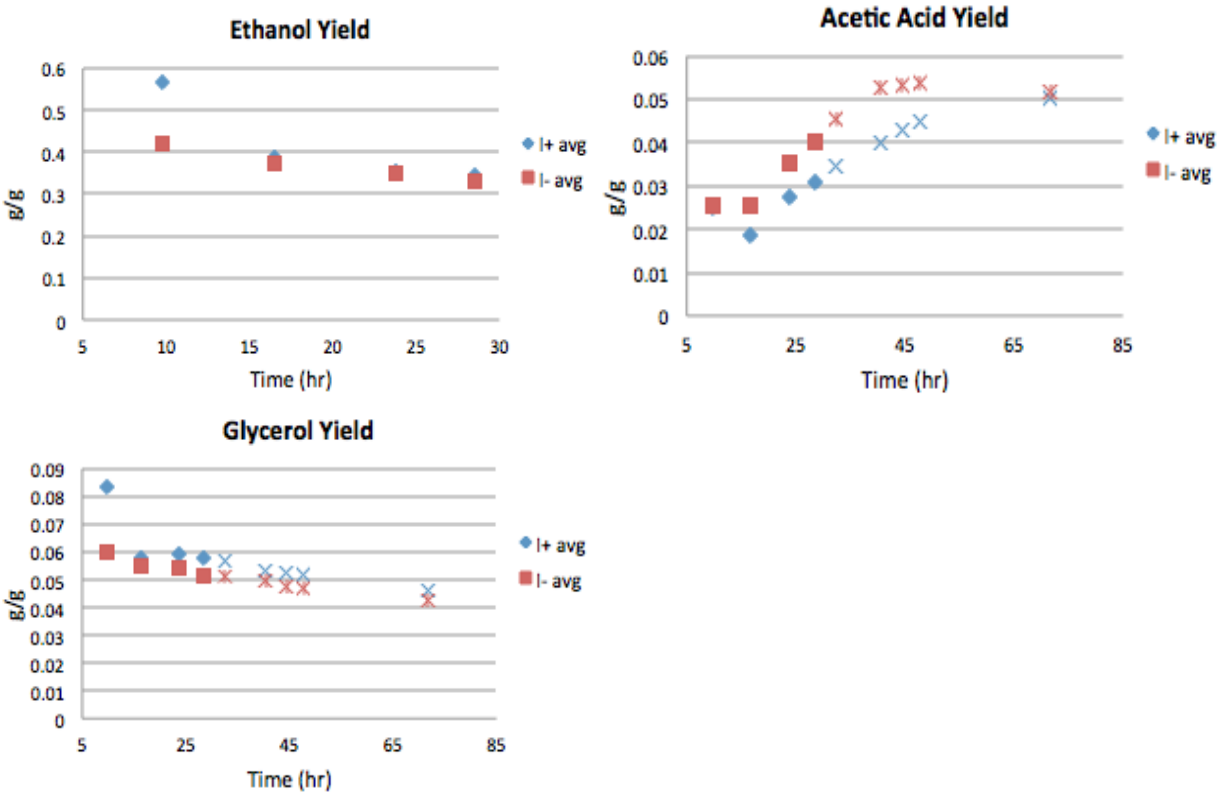



Figure 4. Ethanol, acetic acid, and glycerol yields of *S. cereivisiae* fermentation on I+ and I- under low aeration. The yield calculations for the solid marks were based on glucose consumption whereas the asterisks were based on ethanol consumption up to the time point.

Inositol showed significant effect in lipid composition for *S. cerevisiae*. The lipid quantification in Table 2 showed more total lipid in I+ compared to I-, forming 7.95% and

5.07% of cell mass respectively. Of the storage neutral lipids, SE instead of TAG was the predominate form for both I+ and I-. SE level was 18 folds higher in I+ and 60 folds higher in I-. This phenomenon could be attributed to aeration and the increased in oxygen concentration that favored the pathway of SE synthesis.

Surprisingly, the free fatty acid (FFA) fractions were very high in both I+ and I-, which could indicate that lipids were being degraded. A possible explanation for the occurrence of lipid hydrolysis at 72 hr could be that *S. cerevisiae* cells were still growing from ethanol consumption since around 3 g/L of ethanol were left in both I+ and I- media at 72 hr.

Table 2. Neutral lipid quantification [mg dry lipid weight / g dry cell weight] for *S. cerevisiae* fermentation on I+ and I- media under low aeration.

| (mg/g) | I+ avg | I- avg | I+ avg Standard Error | I- avg Standard Error |
|---|---|---|---|---|
| Total phospholipids | 17.22 | 10.93 | 3.01 | 1.12 |
| Diacylglycerols | 0.93 | 1.68 | 0.05 | 0.80 |
| Free Sterols | 10.09 | 7.22 | 1.80 | 1.18 |
| other | 1.85 | 1.63 | 0.15 | 0.33 |
| other | 2.81 | 0.70 | 0.31 | 0.50 |
| Free Fatty Acids | 7.66 | 4.22 | 0.87 | 0.21 |
| Triacylglycerols | 2.05 | 0.40 | 0.25 | 0.04 |
| Steryl Esters | 36.90 | 23.97 | 5.78 | 1.32 |
| % lipid of cell mass | 7.95 | 5.07 | | |

Since inositol supplementation is known to increase PI synthesis in *S. cerevisiae*, the total phospholipids (PL) composition should be higher in I+ according to Henry, et al [12]. Looking at the lipid composition graphically in Figure 5, our result is consistent with Henry's result, where the total PL in I+ is greater than I- even with substantial error bars. As the result stayed consistent with previous publications, the experiment was used as a control for subsequence assessment of inositol's effect on *Y. lipolytica*.

TAG level was 5-fold higher in I+ than in I- but DAG level was lower in I+ than in I-.

If the dynamics of the metabolism between TAG, DAG, FFA and other lipids were to be determined, it is necessary to asses a kinetic study taking samples at different time points.

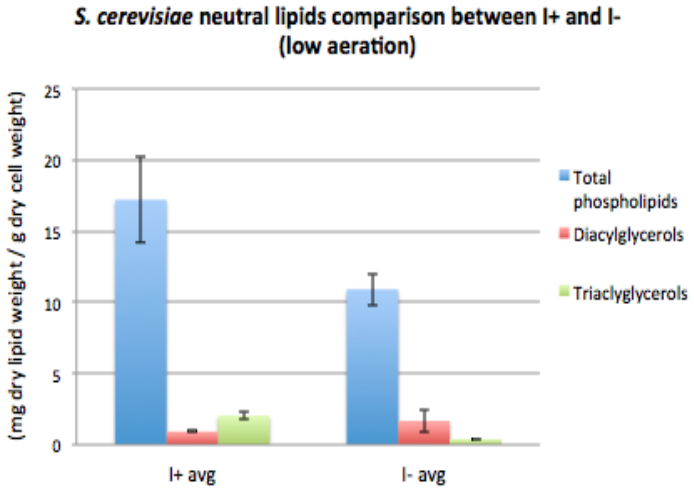
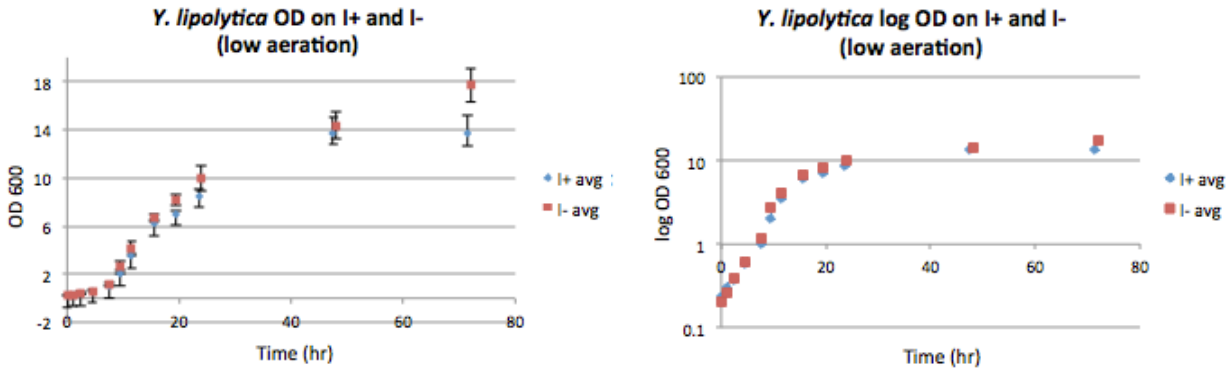



Figure 5. Total phospholipids, DAG, and TAG quantification comparison between *S. cerevisiae* cultured on I+ and I-.

**Experiment 2 - *Y. lipolytica* on I+ I- (aerated 1 SLPM)**

Cell growth for *Y. lipolytica* on low aeration followed sigmoidal growth as shown in Figure 6. Results from fermenter 2 (I+) for all subsequent analysis were disregarded as it was not receiving air due to blocked airway tube. Cell growth for *Y. lipolytica* on I+ and I- media followed similar growth in the beginning until reaching stationary phase. Inositol influenced late cell growth as the average stationary phase OD for I+ was 10.735 (standard error: 0.245) and 12.536 (standard error: 1.012) for I-. Looking at time point 72 hr specifically, the OD were significantly higher for I- compared to I+ according to Figure 6a.




(a) (b)

Figure 6. *Y. lipolytica* on I+ and I- media under low aeration: (a) OD vs time graph (b) logarithmic OD vs time graph.

Specific growth rates for *Y. lipolytica* were summarized in Table 3. I+ and I- had similar specific growth rates: I+ average specific growth rate was 0.2684 $h^{-1}$ and I- average specific growth rate was 0.2752 $h^{-1}$, showing no difference with inositol supplementation. However, these specific growth rates were both higher than that of I+ (0.1899 $h^{-1}$) and I- (0.1945 $h^{-1}$) of a previous experiment of growing *Y. lipolytica* with no aeration. The higher growth rate in this study was due to the low level of aeration provided to *Y. lipolytica,* which is an obligate aerobe.

Table 3. Specific growth rates and standard errors of *Y. lipolytica* on I+ and I- media under low aeration.

| *Y. lipolytica* (low aeration) | I+ avg | I- avg |
|---|---|---|
| μ ($h^{-1}$) | 0.2684 | 0.2752 |
| Standard Error | 0.00685 | 0.0157 |

In Figure 7 below, OD measurements were plotted against cell mass concentrations for *Y. lipolytica* for I+ and I-. A linear relationships existed between cell mass and OD with an $R^2$ values of 0.9994 for I+ and 0.9954 for I-. The high $R^2$ values close to 1 suggested that this graph could be used to determine cell mass from OD measurements for future experiments.

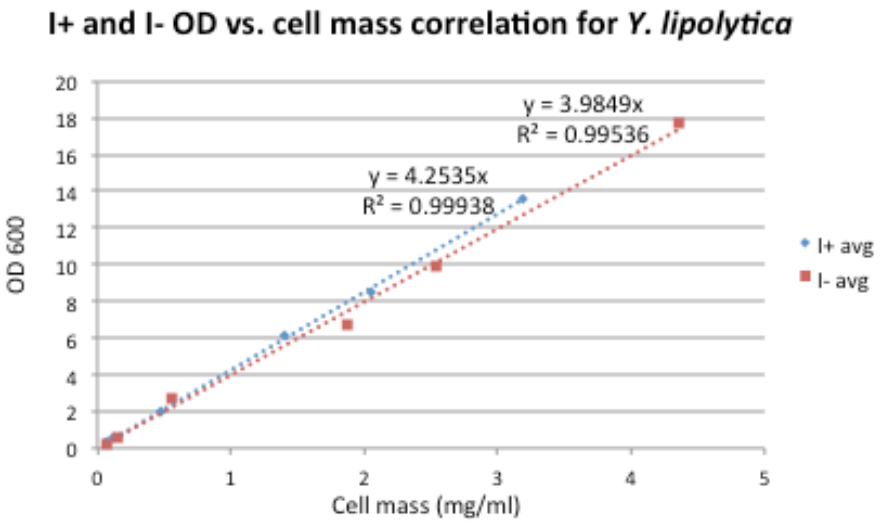



Figure 7. Cell mass correlation with OD for *Y. lipolytica* on I+ and I- under low aeration.

At low aeration, glucose was not completely consumed at the end of 72 hr where 13.62 g/L of glucose was left in I+ and 10.41 g/L of glucose was left in I- shown in Figure 8. More glucose was consumed in I- at 72 hr and the result was reflected in a higher cell growth/OD of *Y. lipolytica* in I- at 72 hr, which was mentioned in the previous section.

A possible explanation for excess glucose left over could be oxygen was the limiting substrate when fermentation was conducted at low aeration at 1 SLPM. As an obligate aerobe, *Y. lipolytica* was not able to fully grow and consume the glucose due to such low aeration rate.

Even though citric acid is a known metabolite for Y. lipolytica, concentration was not quantified in this experiment since the citric acid peak was too small for accurate quantification.

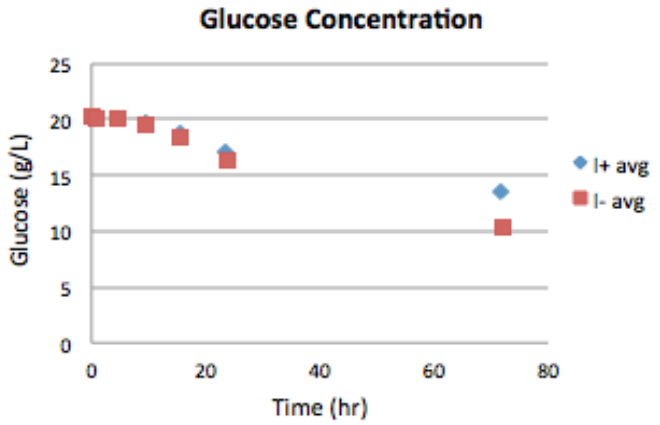



Figure 8. Glucose concentration of *Y. lipolytica* fermentation in I+ and I- on media under low aeration.

The lipid quantification between I+ and I- for *Y. lipolytica* grown under low aeration in Table 4 showed that the lipid compositions were almost completely identical. Therefore, inositol did have an effect on *Y. lipolytica*'s lipid metabolism. The speculation that inositol may affect lipid storage due to the up regulation of SAC1 gene was shown to not have significant impact experimentally.

Scarce amount of lipids were stored in the cells at 72 hr as the total lipids only represented 1.77% of cell mass in I+ and 2.38% of cell mass in I-. The percentages of the total lipids were substantially low, even lower than those of *S. cerevisiae*, which was unusual based on the known fact that *Y. lipolytica* can store lipid of at least 25% of their dry mass [6]. Again, the low aeration could be a possible reason for low lipid accumulation due to low consumption of glucose.

Table 4. Neutral lipid quantification [mg dry lipid weight / g dry cell weight] for *Y. lipolytica* fermentation on I+ and I- under low aeration.

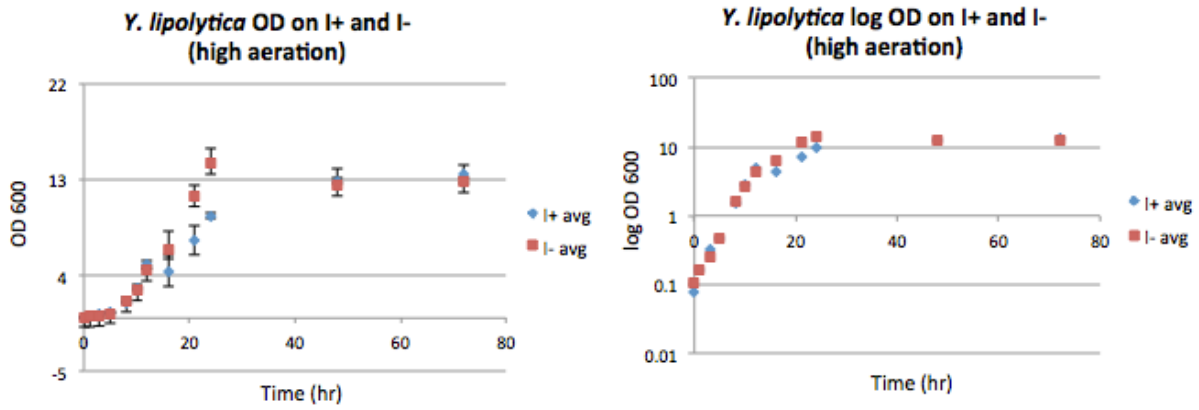
| (mg/g) | I+ avg | I- avg | I+ avg Standard Error | I- avg Standard Error |
|---|---|---|---|---|
| Total phospholipids | 2.29 | 2.99 | 0.67 | 0.18 |
| Diacylglycerols | 0.47 | 0.46 | 0.13 | 0.08 |
| Free Sterols | 1.94 | 6.43 | 0.52 | 1.01 |
| other | 0.25 | 0.23 | 0.08 | 0.06 |
| other | 0.41 | 0.92 | 0.11 | 0.18 |
| Free Fatty Acids | 2.19 | 2.30 | 0.28 | 0.44 |
| Triacylglycerols | 3.16 | 2.30 | 0.87 | 0.39 |
| Steryl Esters | 6.98 | 8.73 | 1.71 | 1.51 |
| % lipid of cell mass | 1.77 | 2.38 | | |

**Experiment 3 - *Y. lipolytica* on I+ I- (aerated 8 SLPM)**

Major water loss (approximately 600 mL in total) was observed towards the end of the fermentation run in all fermenters, which was common for laboratory scale fermenter operating under high aeration for prolong duration [15]. This would result in errors in the OD measurement and HPLC and TLC quantification as they were determined by the amount of culture broth [15]. To compensate for this error, results for OD, HPLC, and TLC would be modified with a correction factor.

To adjust for water loss, OD was multiplied by correction factors at points after 24 hr since water loss was most significant during stationary phase. At 24 hr, about 300 mL of water was lost from an original media volume of 1000 mL and a correction factor of 0.7 was used. At 48 hr and 72 hr, about 600 mL of water was lost and a correction factor of 0.4 was used. The corrected OD and its logarithmic scale were plotted in Figure 9. Cell growth for *Y. lipolytica* on I+ and I- media followed similar growth for the 72 hr with no significant difference in stationary OD as seen in Figure 9a. At stationary phase, the average OD for I+

was 11.992 and the average OD for I- was 13.224 with respective standard errors of 0.414 for I+ and 0.939 for I-.




(a) (b)

Figure 9. *Y. lipolytica* on I+ and I- media under high aeration: (a) corrected OD vs. time graph (b) corrected logarithmic OD vs. time graph.

I+ and I- had similar average specific growth rates of 0.3061 $h^{-1}$ and 0.2853 $h^{-1}$ respectively as shown in Table 5. The difference in specific growth rates on I+ and I- was not significant, indicating inositol had no effect on growth rates. These specific growth rates were higher than the I+ (0.2684 $h^{-1}$) and I- (0.2752 $h^{-1}$) specific growth rates of experiment 2 under low aerating (1 SLPM) condition.

Table 5. Specific growth rates and standard errors of *Y. lipolytica* on I+ and I- under high aeration.

| *Y. lipolytica* (high aeration) | I+ avg | I- avg |
|---|---|---|
| μ ($h^{-1}$) | 0.3061 | 0.2853 |
| Standard Error | 0.00536 | 0.0158 |

With unadjusted HPLC data, the glucose concentration increased drastically from 15.13 g/L at 48 hr to 17.28 g/L at 72 hr for I+ samples and from 14.96 g/L at 48 hr to 17.87 g/L at 72 hr for I- samples. The increase in glucose concentration at the end of steady state is likely due to the 600 mL water loss. Hence, the same procedure used to correct for the last three OD measurements was done on the last three HPLC data points. The corrected glucose concentration was shown in Figure 10 below.

Under high aeration, the final glucose concentration dropped to 6.91 g/L for I+ and 7.15 g/L for I- at 72 hr. Theses glucose concentrations were lower than the glucose level left at the end of the *Y. lipolytica* fermentation under low aeration. *Y. lipolytica* was able to metabolize the carbon source more effectively under higher aeration.

However, even after 72 hr under high aeration, glucose was still not completely consumed. Increasing aeration further in the future for glucose utilization would not be a feasible way unless the volume of water lost can be calculated and replenished throughout fermentation.

*Y. lipolytica* is known to be inefficient at utilizing sugar since it had lower lipid yield and lower growth rate on glucose and xylose, and prefers glycerol over glucose [1]. Papanikolaou's experiment showed that *Y. lipolytica* grown in media containing either equal carbon mol of glycerol or glucose resulted with complete glycerol consumption by 100 hr while there was still half the amount of glucose left [16]. Possible solutions for improving sugar-utilization could be done through genetic engineering and co-

fermentation strategies [1]. A leucine and uracil auxotrophic strain, devoid of secreting extracellular protease activity for hydrophobic substrate utilization, consumed 80g/L of glucose in 70 hr [9]. Co-fermentation on 10 g/L of glucose and 10 g/L of glycerol decreased total sugar consumption time by 25% as compared to fermentation done solely on 20 g/L of glucose [19].

The concentration of citric acid was not quantified in this experiment as well since it was almost close to zero.

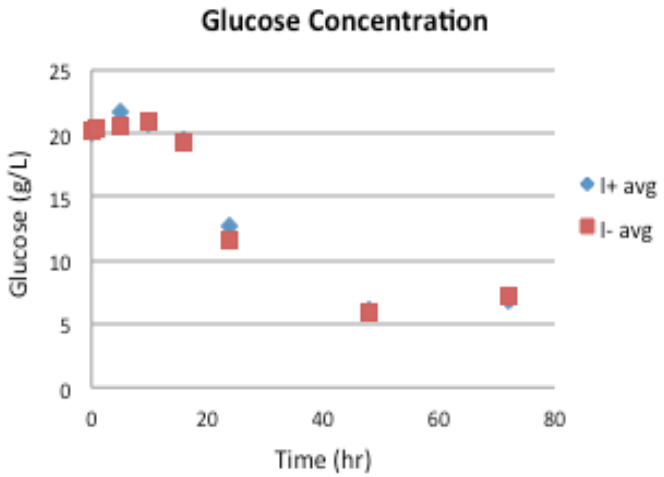



Figure 10. Corrected glucose concentration of *Y. lipolytica* fermentation on I+ and I- media under high aeration.

The corrected lipid quantification was shown in Table 6. The 0.4 correction factor was applied to find the corrected mg/mL of lipid at 72 hr. The correct dry cell weight (g/L) was obtained from mapping the corrected OD at 72 hr onto the cell mass vs OD correlation curved obtained from experiment 2. Dividing the corrected mg/mL of lipid by the correct g/L of cell weight, mg dry lipid weight / g dry cell weight was calculated.

In Table 6, the lipid quantification for *Y. lipolytica* grown under high aeration showed that lipid composition was almost identical between samples with or without inositol supplementation. This again confirmed that inositol does not have an effect on lipid metabolism for *Y. lipolytica*. However, the total lipid quantity drastically increased from lipid representing 1 to 2% of dry cell mass under low aeration to 7.21% in I+ and 7.18% in I- under high aeration. The high level of lipid accumulation could be attributed to the increased glucose consumption when *Y. lipolytica* received enough oxygen. The fraction of FFA was surprisingly high at 72 hr when cells were assumed to enter deep stationary phase. This was possible as Beopoulos et al. indicated that the lipid body of *Y. lipolytica* is capable of accommodating FFA in addition to the known conventional storage lipids such as TAG and SE [8]. The presence of high level FFA is also beneficial for biodiesel production.

Table 6. Corrected neutral lipid quantification [mg dry lipid weight / g dry cell weight] for *Y. lipolytica* fermentation on I+ and I- under high aeration.

| (mg/g) | I+ avg | I- avg | I+ avg Standard Error | I- avg Standard Error |
|---|---|---|---|---|
| Total phospholipids | 9.66 | 9.62 | 1.00 | 1.07 |
| Diacylglycerols | 0.12 | 0.15 | 0.05 | 0.02 |
| Free Sterols | 10.54 | 10.31 | 0.72 | 1.25 |
| other | 0.83 | 0.87 | 0.44 | 0.00 |
| other | 4.49 | 4.39 | 0.09 | 0.70 |
| Free Fatty Acids | 14.01 | 17.32 | 1.33 | 2.75 |
| Triacylglycerols | 12.95 | 13.39 | 0.62 | 1.36 |
| other | 0.55 | 0.19 | 0.07 | 0.12 |
| Steryl Esters | 18.93 | 15.58 | 1.17 | 0.15 |
| % lipid of cell mass | 7.21 | 7.18 | | |

[1] Excluding fermenter 2 (I+) and fermenter 4 (I-) since those samples were accidentally lost.

**Experiment 4 - *Y. lipolytica* on I+ (aerated 10 SLPM and pH control)**

Since this experiment with pH control was only used to check *Y. lipolytica*'s ability to produce citric acid as no citric acid was detected in experiment 2 and 3, only OD and HPLC measurements were taken for a few time points in experiment 4 on I+ media. Specific growth rate calculation and TLC were not performed either.

Major water loss was again noticed under high aeration. A correction factor of 0.5 was used for the assumption that 750 mL water was lost from original media volume of 1500 mL during steady state at 42 hr and 72 hr time points with Figure 11 showing the corrected OD. The assumption that 750mL of water was lost at 10 SLPM was based on linear scale up from the lost of 600 mL of water at 8 SLPM.

The Lag phase was longer because aeration was at 1 SLPM in the beginning of the experiment and later increased to 10 SLPM. In Figure 11, *Y. lipolytica* grew to a maximum average OD of 31.488 on I+ at 72 hr, which was 2.3 times higher than the average OD of 13.525 on I+ at 72 hr obtained from experiment 3. Even though the aeration was a little higher for experiment 4 compared to experiment 3, the great increase in OD/cell growth in experiment 4 should be attributed to the control of optimal cell growth pH at 4.5-5.5.

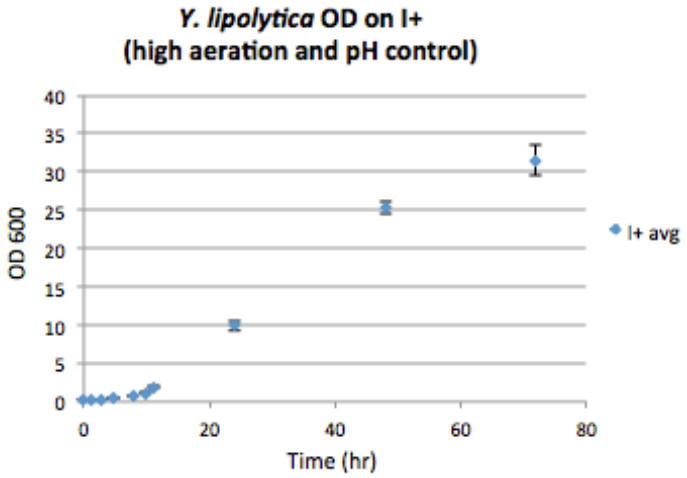
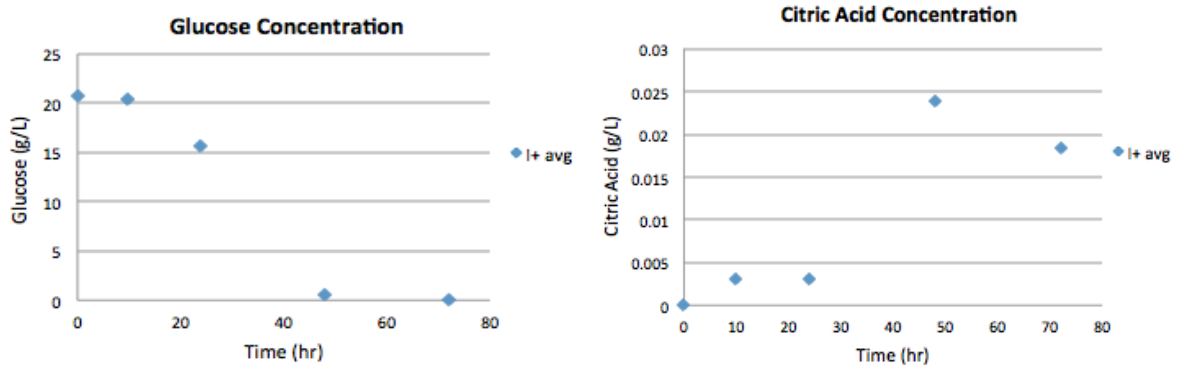



Figure 11. Corrected OD of *Y. lipolytica* on I+ and I- media under high aeration and pH control.

The adjustment used to correct for water loss on OD data was also done on the HPLC data for the last two data points. The concentrations of glucose and citric acid shown in Figure 12 were corrected values. As shown in Figure 12a, glucose was completely consumed at the end of 72 hr fermentation when pH was controlled for optimal growth. The complete consumption of glucose correlated with a maximal OD that reached 31.488, higher than that of experiment 3.

Citric acid concentration was quantified in this experiment as the HPLC software could accurately detect and measure the citric acid produced. According to Figure 12b, a maximal amount of citric acid, 0.024 g/L, was produced at 48 hr. However, this amount was still substantially low compared to several other studies, showing up to 1-5 g/L of citric acid production at ~32 hr [9,17]. These studies cultured *Y. lipolytica* under different media for optimal citric acid production, suggesting that a tailored medium composition should be done if more citric acid production is desired for our strain.




(a) (b)

Figure 12. (a) Corrected glucose and (b) corrected citric acid concentration from *Y. lipolytica* fermentation on I+ and I- under high aeration and pH control.

**DISCUSSION**

1. **Comparison of specific growth rates between *S. cerevisiae* and *Y. lipolytica* under low aeration (1 SLPM)**.

Since experiments for *S. cerevisiae* and *Y. lipolytica* under low aeration (1 SLPM) both resulted in no significant difference between I+ and I- specific growth rates, growth rates for all fermenters in each experiment were averaged. The average specific growth rate for *S. cerevisiae* was 0.2345 $h^{-1}$ and the average specific growth rate for *Y. lipolytica* was 0.2725 $h^{-1}$, which were statistically different (P value = 0.009967, t-test in Appendix II). Thus, *Y. lipolytica* grew faster than *S. cerevisiae* during the exponential growth phase.

2. **Comparison of *Y. lipolycia* specific growth rates under low aeration (1 SLPM) and high aeration (8 SLPM).**

There was no statistical difference between specific growth rates on I+ and I- for both *Y. lipolytica* under low aeration and high aeration. Thus, growth rates for all fermenters were averaged within each experiment. The average specific growth rate for *Y. lipolytica* on low

aeration (1 SLPM) was 0.2725 $h^{-1}$ and the average specific growth rate for *Y. lipolytica* on high aeration (8 SLPM) was 0.2957 $h^{-1}$, which were not statistically different (P value = 0.09811, t test in Appendix II). Even though there was no significant difference between *Y. lipolytica*'s specific growth rates on high versus low aeration, the specific growth rate was still higher under high aeration. Amaral et al. found that specific growth rate of *Y. lipolytica* on YPD medium increases with increasing concentrations of dissolved oxygen [2]. Rywinska et al. also found that *Y. lipolytica* had a higher μ with higher aeration [18]. The published results of higher aeration causing higher μ were reflected in our experiment.

3. **Lipid composition comparison across experiments.**

Figure 13 contained the TAG, SE and total PL levels among the three experiments of growing *S. cerevisiae* at low aeration (1 SLPM), *Y. lipolytica* at low aeration (1 SLPM), and *Y. lipolytica* at high aeration (8 SLPM). Comparing Figure 13a & b, *S. cerevisiae* stored most of its lipid in the form of SE instead of TAG while *Y. lipolytica* stored its lipid as TAG and SE in comparable amount under low aeration, and more in the TAG form under high aeration.

Growing *Y. lipolytica* under low aeration had the least combined storage lipid (TAG and SE) suggesting that future experiment should be conducted at aeration higher than 1 SLPM. Growing *Y. lipolytica* under high aeration had the most TAG among the three experiments. *Y. lipolytica* demonstrated the ability to naturally accumulate high amount of storage lipid in the form of TAG. Unlike *S. cerevisiae*, the PL level of *Y. lipolytica* was not significantly affected by inositol seen in Figure 13c, showing that the two yeasts have different metabolic network for PI synthesis.

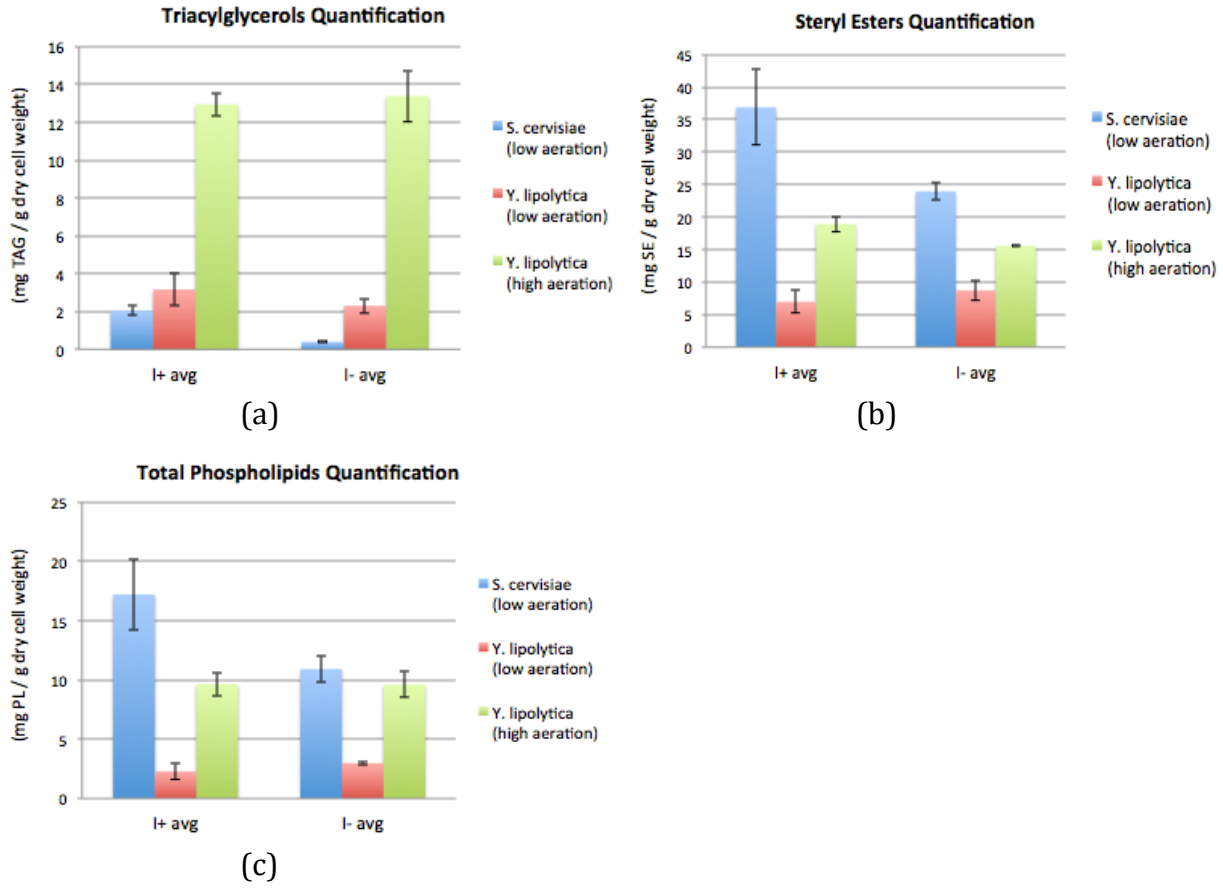



Figure 13. Lipid levels of TAG, SE, and total PL between *S. cerevisiae* at low aeration, *Y. lipolytica* at low aeration, and *Y. lipolytica* at high aeration on I+ and I- media. The graphs for *Y. lipolytica* at high aeration were all corrected lipid values.

4. **Citric acid production comparison across *Y. lipolytica* experiments.**

The production of citric acid by *Y. lipolytica* increased as the rate of aeration increased. The different chromatographs in Figure 14 corresponded to citric acid concentration at 72 hr for experiment 2, 3, and 4. The peak areas shown below in Figure 14 were not adjusted for the water lost that occurred in experiment 3 and experiment 4 from high aeration. A visual comparison can still be made for the first peak of all graphs which correspond to the citric acid concentration, keeping in mind that the peaks for experiment 3 (high aeration) would be 0.4 of the original size and the peak for experiment 4 (higher aeration pH control) would

be 0.5 of the original size. Even though there was not much difference in citric acid concentration between I+ and I- for each experiment, citric acid production varied among the experiments. As aeration increased from experiment 2 to 3 to 4, more citric acid was produced. The increase in dissolved oxygen in the culture is known to improve the yield of secondary metabolites, such as citric acid, which is produced in the citric acid cycle that requires oxygen [11].

The citric acid production was maximized for *Y. lipolytica* under higher aeration with pH control. Experiment 2 and 3 without pH control had an initial pH of 4-5 that gradually dropped to a pH range of 1-3 at the end of the 72 hr fermentation. However, the pH maintained at 4.5-5.5 for experiment 4 satisfied the pH requirement for optimal citric acid production because the pH was needed to be maintained at 5-7 for effective citric acid production according to Yalcin et al [20].

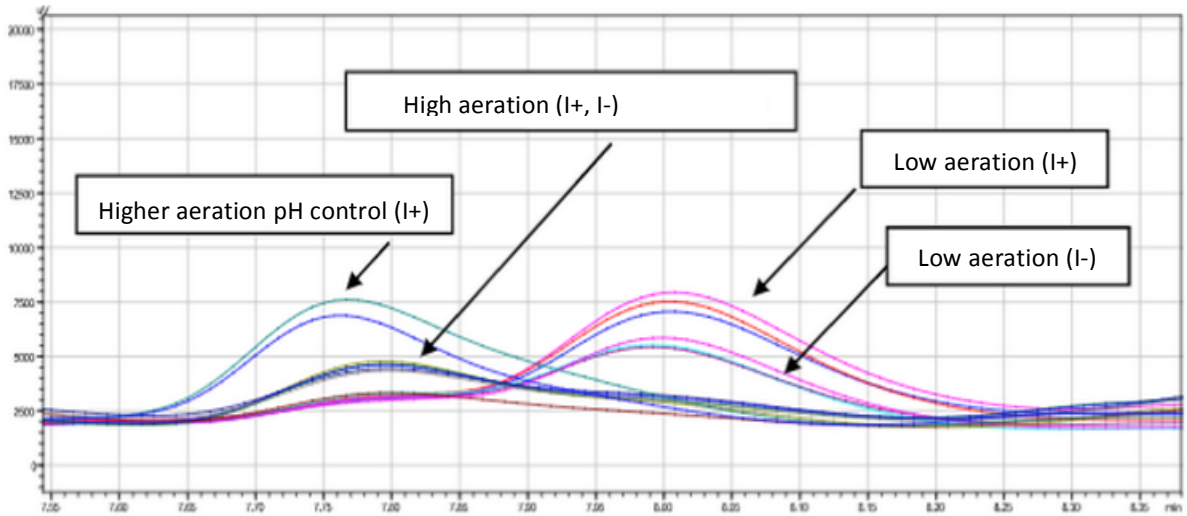



Figure 14. HPLC chromatographs for fermentation samples collected at 72 hr from *Y. lipolytica* grown on I+ and I- under low aeration (experiment 2), high aeration (experiment 3), and higher aeration with pH control (experiment 4). The first elution peak of all graphs at 7.76 min corresponded to citric acid concentration detected by UV detector. The second peak seen in the samples from the low aeration experiment was unknown metabolite.

**CONCLUSION**

This study showed that inositol supplementation did not have significant affect on lipid compositions of *Y. lipolytica* under both low and high aeration. As for other key parameters, inositol had no effect on the growth rates of both *S. cerevisae* and *Y. lipolytica*. However, inositol influenced the stationary OD for *S. cerevisiae* and *Y. lipolytica* under low aeration. Inositol also influenced organic acid and lipid production of *S. cerevisiae* and the glucose consumption of *Y. lipolytica* under low aeration.

Even though inositol did not directly have an effect on *Y. lipolytica*'s lipid composition, inositol supplementation decreased the glucose consumption at the end of stationary phase, showing inositol's influence on other parts of the metabolic network. Multiple replications could be performed in the future to further validate the effects of inositol on *Y. lipolytica*. A higher concentration of inositol supplementation, greater than 75 µmol, could also be investigated to ensure that enough inositol is left for cell to metabolize after prolong fermentation time.

Since inositol supplementation was concluded to not have a significant effect on *Y. lipolytica's* storage lipids from this study, future work in maximizing TAG production could be done on media tailored for optimizing *Y.* lipolytica's growth and lipid synthesis. The media used in this study, which was a media recipe tailored for *S. cerevisiae,* should be modified since the metabolic network is different between S*. cerevisiae* and *Y. lipolytica*, which is shown through the results of this fermentation study. A suggestion for media manipulation is to adjust the nitrogen and carbon concentration. The Kennedy pathway is

induced by growing *Y. lipolytica* in low nitrogen concentration since it causes the formation of excess cytosolic acetyl-CoA to be incorporated into TAG [6,7]. Since cells need nitrogen for essential protein synthesis during growth phase, nitrogen restriction should be placed during the stationary phase. With the C/N ratio of 20 Cmol $Nmol^{-1}$ applied during stationary phase, higher level of lipid body was observed in the experiment from Beopoulos et al. [5]. Future experiments should be conducted based on tailored media with pH control starting at 1 SLPM aeration with increased aeration rate. Instead of media or growth condition manipulation, perhaps another more effective way of inducing TAG synthesis is to genetically engineer strains that direct carbon flux towards TAG synthesis.

This comprehensive fermentation study provides basic insights on inositol's effect on the metabolism of *S. cerevisiae* and *Y. lipolytica*. These baseline results can be used as comparison for future experiments that try to optimize key parameters such as cell growth, specific growth rate, and metabolite production. As stated, it is important to understand the consumption, production, and accumulation of certain carbohydrates and metabolites in yeasts fermentation in order to exploit their respective metabolic network for biotechnology applications such as fuels and bioproducts.

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**Appendix I.**

Recipe for 1 L of medium

- Add 20 g of dextrose (=glucose)
- Add 5.0 g of ammonium sulfate $(NH_4)_2SO_4$
- Add **YNB salts-Henry Lab's synthetic mix**, which consist of:

0.982 g Potassium phosphate [anhydrous, monobasic ($KH_2PO_4$)]
0.491 g Magnesium sulfate [anhydrous ($MgSO_4$)]
0.0982 g Sodium chloride [anhydrous (NaCl)]
0.130 g Calcium chloride [$CaCl_2 . 2H_2O$]

- Add **Trace Components**, which consist of:

0.543 mg Boric acid ($H_3BO_3$ /Fisher Biotech Cat # BP168-1)
0.0435 mg Cupric sulfate ($Cu\ SO_4 \bullet 5\ H_2O$ /sigma Cat # C-6283)
0.109 mg Potassium iodide (KI / Fisher Cat # P410-100)
0.217 mg Ferric chloride ($FeCl_3 \bullet 6H_2O$ /Sigma Cat # F-1513)
0.435 mg Manganese Sulfate ($MnSO_4 \bullet H_2O$/Sigma Cat # M-7634)
0.217 mg Sodium molybdate ($MoNa_2O_4$/Fisher (Acros) Cat # 20637-0050)
0.435 mg Zinc sulfate ($ZnSO_4 \bullet 7H_2O$ / Sigma Cat # Z4750)

- Add **Vitamins, which consist of:**

0.001996 mg D- biotin
0.3992 mg Calcium pantothenate
0.001996 mg Folic Ac
0.3992 mg Niacin
0.1996 mg p-aminobenzoic Ac (PABA)
0.3992 mg Pyridoxine hydrochloride
0.1996 mg Riboflavin
0.3992 mg Thiamine, HCl

- Add **Complete AA with Ade & Ura:**

0.02 g Adenine sulfate (Fisher/Acros Cat#16363-0250)
0.02 g L-Arginine HCl (Fisher Cat#BP372-100)
0.02 g L-Histidine HCl (Fisher Cat#BP383-100)
0.06 g L-Leucine (Fisher Cat#BP385-100)
0.23 g L-Lysine HCl (Fisher Cat#BP386-100)
0.02 g L-Methionine (Fisher Cat#BP388-100)
0.3 g L-Threonine (Fisher Cat#BP394-100)
0.02 g L-Tryptophan (Fisher Cat#BP395-100)
0.04 g Uracil (Sigma Cat#U0750)

- For I+ media, add 0.0135 g of *myo*-inositol (from Sigma Cat # I-5125)

For preparation of liquid media bring volume up to 1L with $dH_2O$
For preparation of Plates bring the volume to 980 mL with $dH_2O$ and then add 20 g of agar

## Appendix II.

Difference between growth rates of *S. cerevisiae* and *Y. lipolytica* under low aeration (1SLPM)
t-Test: Two-Sample Assuming Unequal Variances with the Level of Significance of 0.05

| | *S. cerevisiae* μ | *Y. lipolytica* μ |
|---|---|---|
| Mean | 0.2345 | 0.2725 |
| Variance | 0.000118 | 0.000406 |
| Observations | 5 | 5 |
| Hypothesized Mean Difference | 0 | |
| df | 6 | |
| t Stat | -3.71021 | |
| P(T<=t) two-tail | 0.009967 | |
| t Critical two-tail | 2.446912 | |

Difference between *Y. lipolytica* growth rates under low aeration (1SLPM) and high aeration (8SLPM)
t-test: Two-Sample Assuming Unequal Variances with the Level of Significance of 0.05

| | *low aeration* μ | *high aeration* μ |
|---|---|---|
| Mean | 0.2725 | 0.2957 |
| Variance | 0.000406 | 0.000464 |
| Observations | 5 | 6 |
| Hypothesized Mean Difference | 0 | |
| df | 9 | |
| t Stat | -1.84512 | |
| P(T<=t) two-tail | 0.09811 | |
| t Critical two-tail | 2.262157 | |