THERMUS THERMOPHILUS

AS A THERMOPHILIC

MODEL ORGANISM FOR

BIOFUELS PRODUCTION

by

Jing Lu

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Degree in Chemical Engineering with Distinction

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ABSTRACT

The potential of using *Thermus thermophilus* HB8, a thermophilic bacterium, for the production of biofuels was investigated in this work. *T. thermophilus* is a thermophilic bacterium that thrives naturally at high temperatures, typically around 70-85°C. This bacterium has significant potential in the field of biotechnology because of its unique physical and biochemical properties, especially for the production of low-boiling biofuels. In this project, the growth of *Thermus thermophilus* HB8 was characterized in batch cultures under defined experimental growth conditions.

First, we optimized a defined growth medium to maximize the growth rate of *T. thermophilus* without the need to use yeast extract in the medium. After that, growth rates were measured under aerobic growth conditions for temperatures ranging between 50°C and 90°C with glucose as the only carbon source. The optimum growth temperature was 80°C and the maximum growth rate of *T. thermophilus* was 0.28 1/hr, which corresponds to a doubling time of about 2.5 hours. In the growth experiments, *T. thermophilus* was cultured in custom constructed mini-bioreactors (at 10-mL scale) that were constructed using Hungate tubes and septum caps. For the growth experiments, we used uniformly ^{13}C-labeled [U-^{13}C]glucose as the carbon source and measured $^{13}CO_2$ production in the off-gas by an on-line mass spectrometer (at *m/z* 45). With this setup we followed the growth in real-time. In addition to measuring CO_2 production, we also measured glucose concentration and optical density at 600 nm (OD_{600}) during the cultures.

From all of these measurements, we calculated growth rates for the exponential growth phase. The growth of these cells was then further examined for the potential of

producing biofuels by adding various alcohols (ethanol, n-butanol, isobutanol, and propanol) and measuring cell growth. From this data we determined alcohol tolerance for the different potential biofuels. Finally, we applied directed evolution to improve the growth of *T. thermophilus* HB8. The final adapted cells (termed *T. thermophilus* JL3 and JL4) displayed higher growth rate, higher tolerance to glucose, higher concentrations of ethanol, and grew to higher cell densities compared to the starter strain *T. thermophilus* HB8.

Chapter 1

INTRODUCTION

1.1 Motivation

With the depleting supply of petroleum fuel and increasing global fuel demands, biofuels production using microorganisms has become an attractive alternative. In the past decades, microorganisms such as *E. coli* and *S. cerevisiae* have been extensively studied and reengineered by scientists to produce biofuels and chemicals [1]. The conversion of cellulosic biomass to ethanol has been among the leading alternatives to petroleum derived fuels [7]. Scientists have also successfully produced other biofuels and chemicals using these microorganisms. A major consideration of producing biofuels using these microorganisms is the cost of treatment processes. Despite reduced cost of cellulolytic enzymes in recent years, sugar released from biomass still remains an expensive and slow step. This is where thermophilic bacteria have a potential advantage [2]. Thermophilic bacteria thrive at high temperatures, above 70°C. Cellulolytic thermophiles such as *Thermus thermophilus* could be used in conjunction to hydrolyze and ferment sugars from renewable biomass resources to valuable products or consolidated bioprocessing. The use of these microorganisms could potentially lower the quantity of added cellulose required, and hence, significantly reduce production cost. The full potential of biofuels production from cellulosic biomass should be obtainable in the next 10 to 15 years [8].

1.2 *Thermus thermophilus*

T. thermophilus is a gram negative eubacterium with an optimal growth temperature ranging between 70 and 85 degrees Celsius (i.e. depending on the specific strain)[5]. A major consideration for economical production of biofuels using microorganisms is the cost of product separation. The recovery cost (by distillation) of the final product from the fermentation broth is significant. Thermophilic bacteria have a potential advantage [2]. Thermophiles could be used in conjunction to hydrolyze and ferment all sugars. This would greatly lower the quantity of added cellulose required, and hence, also significantly reduce processing costs.

Many microorganisms that grow at high temperatures, such as *Thermus thermophilus* HB8, utilize a variety of carbohydrates pertinent to the conversion of biomass to biofuels. Many extremely thermophilic enzymes can be identified in the genome sequences. Characterization of these enzymes will require intensive effort but is likely to generate new opportunities for the use of renewable resources as biofuels [4]. *Thermus thermophilus* HB8 is one of these important organisms for producing biofuels. *T. thermophilus* is relatively easy to genetically engineer and hence has the potential to become a model organism for biorefinery applications [5].

1.3 Previous work

T. thermophilus HB8 was used in this study. Previous studies have been done to determine the optimal growth medium for this genus. However, more studies needed to be conducted to determine the best medium composition for *T. thermophilus* HB8 specifically. In previous work in the Antoniewicz lab, performed by Aditi Swarup, glucose and yeast extract concentrations were varied to determine optimal concentrations for growth. The objective was to find a defined medium composition that contains as little yeast extract as possible, since

yeast extract introduces unknown carbons, dilutes amino acid labeling, and increases fermentation cost.

1.4 Aim and outline of thesis

The objective of this thesis was to determine a defined medium that contains as little yeast extract as possible and maximizes biomass density and glucose utilization for efficient cell growth. Chapter 2 outlines the methodology involved in performing the cell culture experiments. Chapter 3 provides details on the results of the cell culture experiments.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

20 wt% Glucose solution, and 1 % yeast extract solution were prepared in distilled water and filter sterilized. Wolfe's minerals and vitamins were obtained from ATCC (Manassas, VA). In addition, 50 mg/mL NH_4Cl solution, and 20 wt% [U-^{13}C]glucose solution were prepared. *T. thermophilus* HB8 (ATCC 27634) was used in this study.

Defined medium MRA1 contained (per liter of medium): 0.50 g K_2HPO_4, 0.30 g KH_2PO_4, 0.50 g NH_4Cl, 0.50 g NaCl, 0.10 g $MgSO_4.7H_2O$, 0.006 g $CaCl_2.2H_2O$, 0.10 g Na_2SO_4, and 4.8 g Tris buffer. The pH was adjusted to 7.7. Glucose, yeast extract, Wolfe's minerals and vitamins were added as indicated in the text.

Defined medium MRA2 contained (per liter of medium): 0.50 g K_2HPO_4, 0.30 g KH_2PO_4, 0.50 g NH_4Cl, 0.50 g NaCl, 0.20 g $MgCl_2.6H_2O$, 0.04 g $CaSO_4.2H_2O$, and 4.8 g Tris buffer. The pH was adjusted to 7.7. Glucose, yeast extract, Wolfe's minerals and vitamins were added as indicated.

2.2 Miniature Bioreactors

A set of mini-bioreactors were constructed for growing *T. thermophilus*. Each mini-bioreactor system consisted of one culture tube as the bioreactor, and one culture tube as the condenser. Figure 2.1 shows the configuration of the mini-bioreactors.

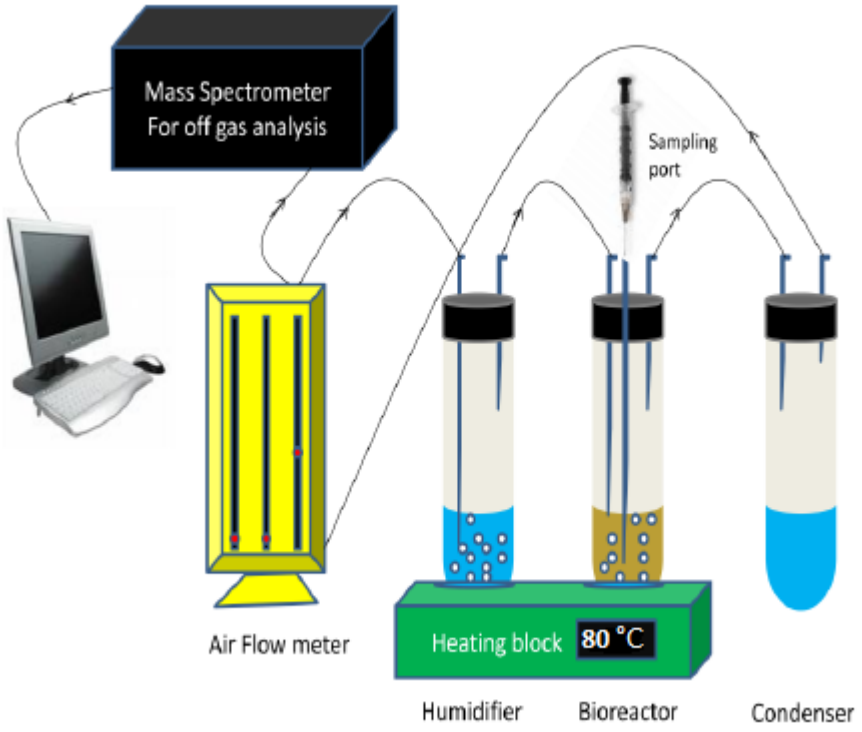

Figure 2.1: Experimental set-up for batch culture of *T. thermophilus*.

Multiple parallel cultures with a working volume of 10 mL were set up in which base medium MRA1 or MRA2 were prepared with different glucose concentrations as indicated. Wolfe's minerals, Wolfe's vitamins, yeast extract solution, and ammonium chloride solution were also added to the medium as indicated. The culture tubes were 15 mL Hungate tubes with a screw cap and a rubber septum that was pierced by needles for gas supply (filtered air), sampling, and gas efflux. Mixing was achieved through the rise of gas bubbles. The condenser

was maintained at room temperature and was used to collect condensed water from the bioreactor off-gas. The mini-bioreactors were autoclaved before each experiment.

2.3 Strain and growth conditions

T. thermophilus HB8 (ATCC 27634) was used in this study. Batch fermentation were conducted in 10 mL miniature bioreactors. The mini-bioreactors were maintained at designated temperatures by placing them in the heating blocks. The growth of these cells was observed for up to 72 hours by taking optical density measurements at wavelength of 600 nm (OD_{600}) using a spectrophotometer and glucose concentrations over time. Samples were obtained using the sampling port shown in Figure 2.1. Prior to taking the glucose concentration measurements, the cells were centrifuged to prevent cell aspiration by the YSI glucose analyzer with a sensitivity of 1%.

2.4 Off-gas Analysis

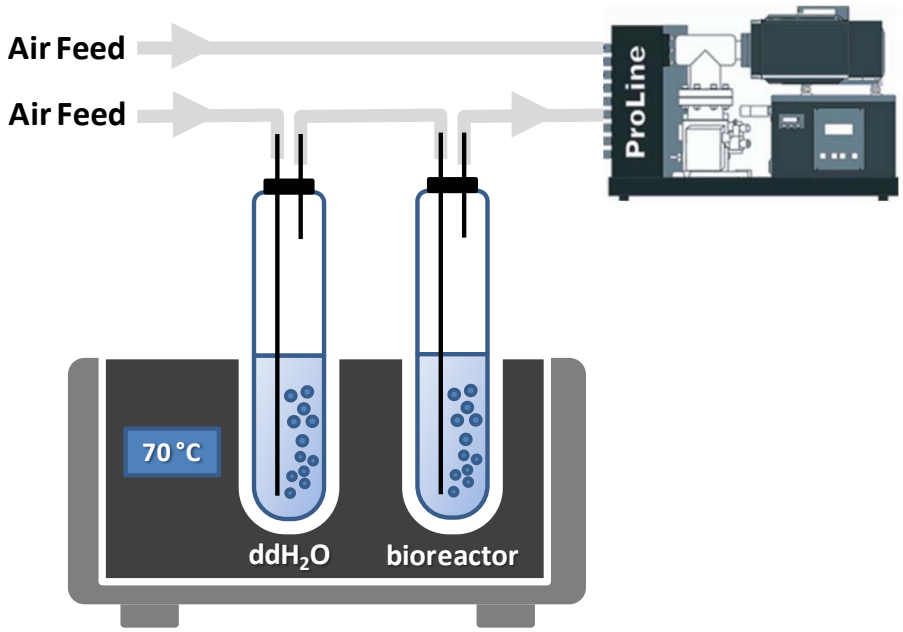

Figure 2.2: Real-time carbon dioxide monotoring by ProLine mass spectrometer.

The inlet air and the off-gas from the mini-bioreactors were directed to a process mass spectrometer for off-gas analysis. Concentrations of nitrogen, oxygen, and carbon dioxide were measured in molar percentages to monitor cell growth. To determine fractional labeling of CO_2 in the off-gas the relative intensities of $^{12}CO_2$ (*m/z* 44) and $^{13}CO_2$ (*m/z* 45) were measured. The set-up of the on-line mass spectrometer is shown in figure 2.2.

2.5 Gas Chromatography-Mass Spectrometry

GC/MS analysis was performed on an Agilent 7890A GC system equipped with a DB-35 MS (30 m x 0.25 mm i.d., 0.25 μm-phase thickness) capillary column, connected to a Waters Quattro Micro Tandem Mass Spectrometer operating under ionization by electron impact (EI) at 70 eV. Helium flow was maintained at 1.0 mL/min via electronic pressure control. The interface temperature was maintained at 250 °C. Intensities of selected ions were recorded using the MassLynx software. Mass isotopomer distributions were obtained by integration[10].

2.6 Derivatization of amino acids

Tertbutyldimethylsilyl (TBDMS) derivatization of biomass amino acids was performed prior to GC/MS analysis. 1 ml samples were taken at the end of the tracer experiments and centrifuged for 5 minutes at 14,000 rpm and the cell pellet and the supernatant were separated. The cell pellet was hydrolyzed with 500 μl of 6N HCl for 18 hours at 110°C. After cooling to room temperature, it was centrifuged at 14,000 rpm for 5 minutes to remove cell debris. The contents were transferred to a new eppendorf tube and evaporated to dryness under air flow at 65°C. The dried sample was dissolved in 50 μl of pyridine and 50 μl of N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA)

+1%TBDMCS was added and the reaction allowed to proceed for 30 minutes at 60°C. The sample was again centrifuged to remove cell debris and finally transferred to a GC injection vial[10].

2.7 GC/MS analysis of amino acids

GC/MS analysis was conducted to quantify the labeling of biomass amino acids. 1 µL of derivatized sample was injected at split ratio of 1:40. The GC oven temperature was held at 80°C for 2 min and then increased at a ramp of 7°C/minute to 280°C. The total run time was 50 minutes. The detector was operated in selected ion recording (SIR). GC/MS analysis was also performed at split ratio of 1:4 to obtain spectra for amino acids with low intensities, namely methionine, histidine, lysine and threonine[10].

2.8 Medium Optimization Experiments

Initially, all cells were grown from frozen stock using a rich medium containing 10 mL of M162 base medium, 1 mL of 1% yeast extract, 50 µL Wolfe's vitamins, 50 µL Wolfe's minerals, and 100 µL of 20% glucose. The cells were grown to an OD_{600} of 1.0 to ensure constant cell density. The media was pre-warmed at 70°C in an incubator before inoculating with 100 µL of cells to the prepared medium.

The growth of *Thermus thermophilus* HB8 was first characterized in batch cultures under defined experimental growth conditions. The first set of experiments was performed with varying glucose concentration to determine the optimal glucose concentration that provides high growth rates for the cells without adding yeast extract. Five parallel cultures were set up with varying glucose additions of 100 µL – 500 µL with increments of 100 µL. Next, experiments were performed to determine the best base medium to use with media MRA1 and MRA2.

2.9 Temperature Optimization Experiments

After the optimal media compositions were determined, experiments were performed to find optimal growth temperature that resulted in the best growth rate. In these experiments, three parallel cultures were set up simultaneously where each contained 10 mL of MRA2, 50 µL of Wolfe's vitamins, 50 µL of Wolfe's minerals, 100 µL of 50 mg/mL NH_4Cl solution. The first culture tube contained 200 µL of 20% glucose solution with 50 µL of 1% yeast extract. The second culture tube contained 100 µL of 20% glucose solution with 50 µL of 1% yeast extract. The third culture tube contained 200 µL of 20% glucose solution with no yeast extract. The experiments were conducted with temperatures of 45°C – 90°C with 5 degrees of increments. Experiments were repeated twice to ensure consistency in the results.

2.10 Effects of supplements experiments

After optimal media compositions and temperature were determined, experiments were performed to study the effects of the various additions of Wolfe's vitamins, Wolfe's minerals, and yeast extract. In these experiments, three or four parallel cultures were set up using the indicated medium. A total of eight experiments were performed. In the first experiment, the amount of vitamins was varied (0, 0.5, and 2%) while everything else was kept constant with no yeast extract. In the second experiment, a similar experiment was performed but with added yeast extract. In the third experiment, the amount of Wolfe's minerals was varied (0, 0.5, and 2%) while everything else was kept constant with no yeast extract. In the fourth experiment, a similar experiment was performed with added yeast extract. In the fifth experiment, the amount of yeast extract was varied (0, 0.01, and 0.02%) while everything else kept constant. In the sixth experiment, the experiment was run with optimal medium with and without yeast extract. In the seventh experiment, the amount of Wolfe's vitamins and Wolfe's minerals were varied simultaneously with 0, 0.5, 1, and 2%

with no added yeast extract. In the eighth experiment, the optimal medium was used with varying amounts of yeast extract of 0, 0.02, 0.1, and 0.2%.

2.11 Alcohol Tolerance Experiments

The next sets of experiments were performed to determine the tolerance of *T. thermophilus* to various biofuels, including the following alcohols: ethanol, n-butanol, propanol, and isobutanol. All experiments were conducted at 80°C. Five parallel culture were set up simultaneously each containing 10 mL of MRA2, 50 µL of Wolfe's vitamins, 50 µL of Wolfe's minerals, 100 µL of 50 mg/mL NH_4Cl solution, and 100 µL of unlabeled glucose. The various alcohols were added after the cells had grown to an OD_{600} of 1.5 to minimize evaporation. The added media each contained 1 mL of MRA2 medium, 100 µL of [U-^{13}C]glucose, and various amounts of alcohol as indicated.

2.12 Adaptive Evolution

In the final set of experiments, adaptive evolution was applied to select for cells with improved growth characteristics. The cells were grown on the same medium for several weeks, until consistent growth was reached by observing the CO_2 production from off-gas analysis. The new cells were named JL1, JL2, JL3, and JL4. For the JL1 and JL2, the growth medium consisted of 10 mL of MRA2, 50 µL of Wolfe's vitamins, 50 µL of Wolfe's minerals, 100 µL of NH_4Cl solution, 200 µL of glucose solution. JL2 contained an extra 400 µL of ethanol. For the JL3 and JL4 cells, all medium compositions were the same as JL1 and JL2 except for the glucose amount used was 400 µL. The new cells were then studied by performing the same glucose experiments that were conducted using the initial strain HB8.

Chapter 3

RESULTS AND DISCUSSION

3.1 Effects of increasing glucose concentration on cell growth

The first set of experiments was done to determine the optimal glucose concentration for growth of *T. thermophilus* HB8. Growth was measured at glucose concentrations of 0.2%, 0.4%, 0.6%, 0.8% and 1.0% (i.e. 2-10 g/L glucose). The mini bioreactors had a working volume of 10 mL and were kept at 70°C. Figure 3.1 shows the OD_{600} measurements and glucose concentrations over time.

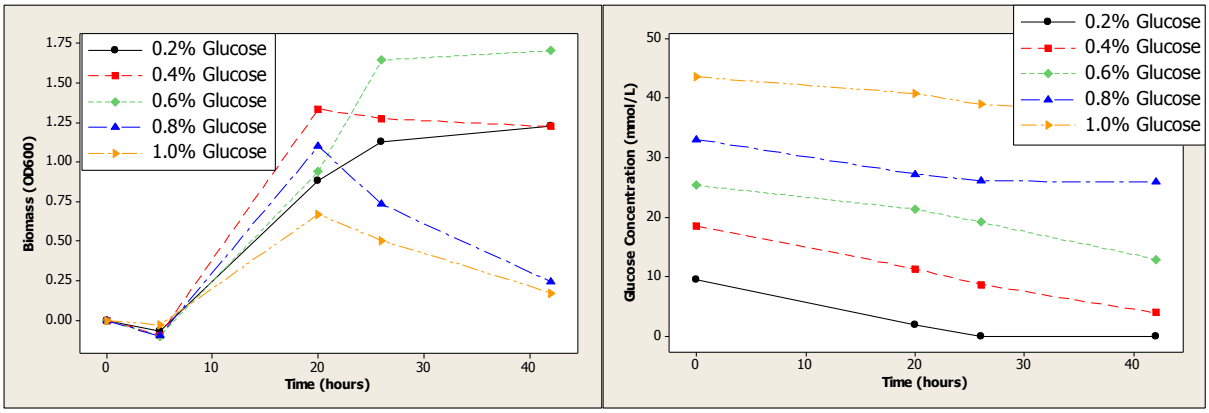

Figure 3.1: Biomass density and glucose consumption of *Thermus thermophilus* HB8 with increasing glucose concentration.

The results indicated that there was almost no growth observed in the reactors with 0.8% and 1.0% glucose concentrations (i.e. 8 g/L and 10 g/L). It was also observed that the cells grew optimally with a glucose concentration of 0.6%. Thus, we concluded that glucose concentrations above 0.6% inhibited growth of cells. The results contradict previous observation that the cells showed no growth above 0.4% of glucose concentration. These

patterns were expected because small amounts of glucose would not give good labeling of amino acids in biomass in ^{13}C-labeling experiments, and very high amounts of glucose could be toxic to cells.

For the next experiment, the same medium compositions were used as the previous experiment, except that we added additional NH_4Cl. *T. thermophilus* HB8 is reportedly able to grow anaerobically under the presence of nitrate due to synthesis of the nitrate reductase complex encoded by the nar operon[6]. In order to maximize cell growth, 100 µL of 50 mg per liter of NH_4Cl solution was added to the medium of each bioreactor. The results show that the cells growing with the ammonium chloride solution reached maximum growth faster than cells growing without the additional ammonium chloride solution. Figure 3.2 shows the biomass density (OD600) and glucose consumption for these experiments.

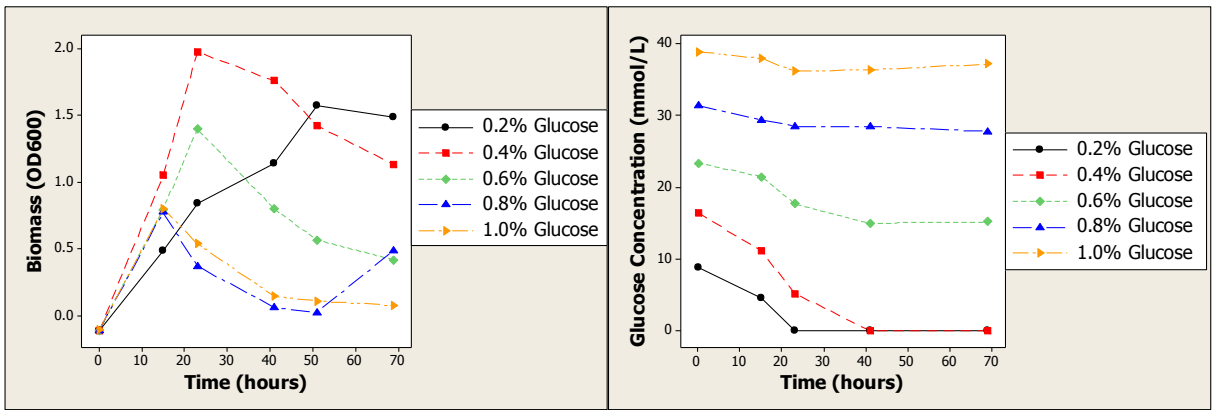

Figure 3.2: Biomass density and glucose consumption of *Thermus thermophilus* HB8 with increasing glucose concentration and added NH_4Cl solution.

3.2 Effect of growth temperature on growth rate

The next set of experiments was done to determine the optimal growth temperature. These experiments were done for temperatures between 50°C and 95°C, with increments of

five degrees. The same medium composition was used for all temperature experiments. Table 3.1 shows the specific amounts of medium compositions.

Table 3.1: Medium compositions for experiment performed at 50 to 95 °C.

Reactor Number	MRA2	Minerals	Vitamins	NH4	Yeast	[U-13C] Glucose
1	10 mL	50 µL	50 µL	100 µL	50 µL	200 µL
2	10 mL	50 µL	50 µL	100 µL	50 µL	100 µL
3	10 mL	50 µL	50 µL	100 µL	-	200 µL

It was expected that the growth rates would increase as the temperature increases until reaching an optimum growth temperature. The growth rates would then decrease, eventually going to zero. A plot of growth rates vs. temperature is shown in figure 3.3. It was observed that the maximum growth temperature was 80°C. The growth rate quickly decreased after reaching the maximum temperature and eventually went to zero at a temperature of 90°C. The growth rates were calculated based on the measured OD_{600} values.

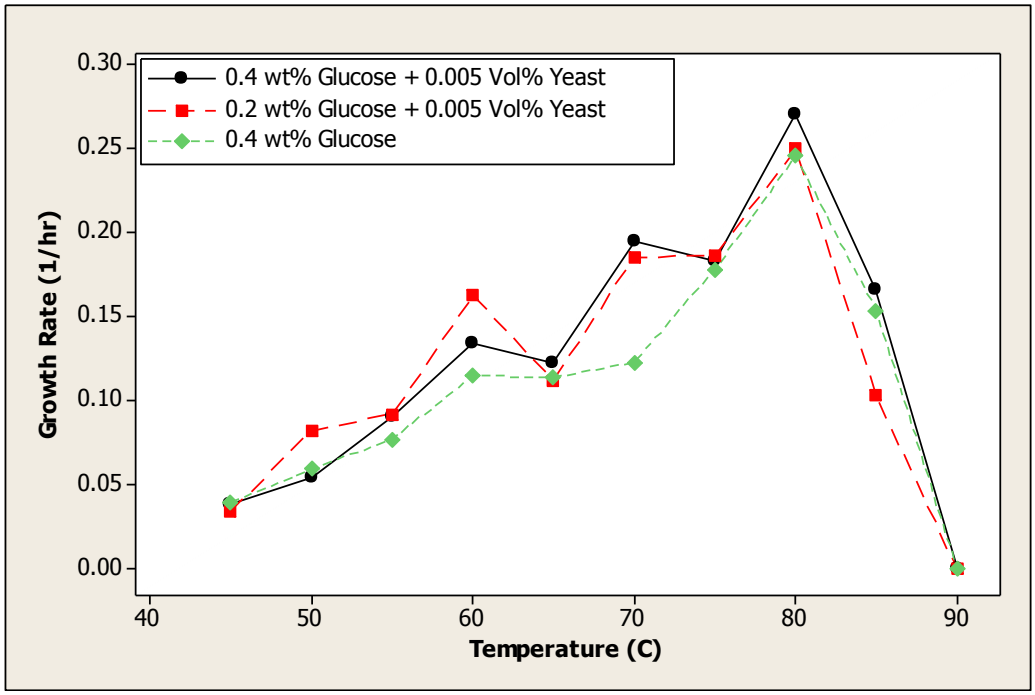

Figure 3.3: Growth rates vs. growth temperatures. Growth temperature profile for 45 to 90 degrees Celsius with five degrees of increment.

3.3 Effects of supplements on growth rate

Next, growth conditions were studied for effects of different amounts of supplements including Wolfe's vitamins, Wolfe's minerals, and yeast extract. These experiments were done at a growth temperature of 80°C. The results show that at high concentrations, these supplements had little impact on cell growth. Plots of biomass density (OD_{600}) vs. time for varying amounts of supplements are shown in figure 3.4. For the experiment with varying amounts of yeast extract, it was observed that the cells reached their maximum OD_{600} value faster with decreasing yeast extract concentration. This was also true for individual Wolfe's minerals and vitamins. However, when vitamins and minerals were increased simultaneously, it was observed that at concentrations higher than 0.5% each, the growth rates were similar. Therefore, it was decided that 0.5 % of vitamins and minerals was the optimum amount.

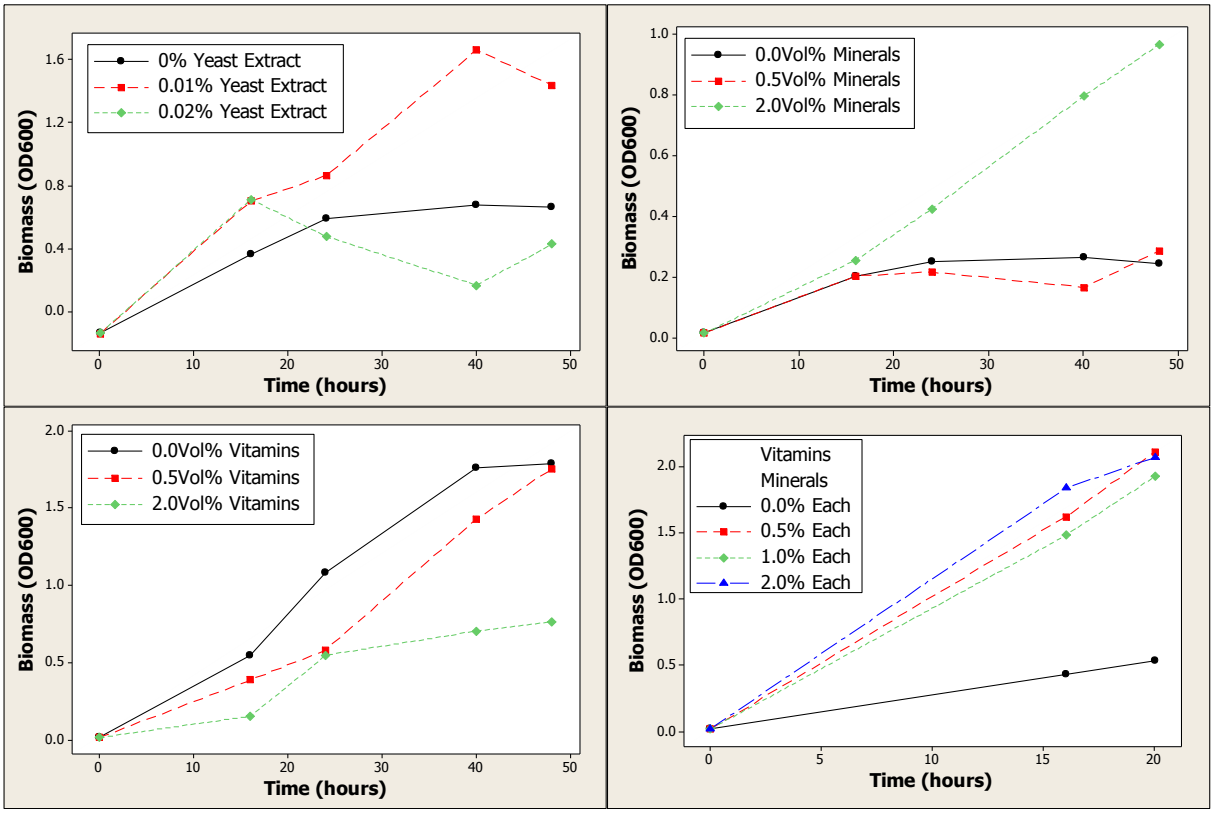

Figure 3.4: Growth of *T. thermophilus* on varying amounts of yeast extract, vitamins, and Wolfe's minerals.

3.4 Biofuels tolerance experiments

In the following set of experiments, the effect of alcohol inhibition was investigated. Several different alcohols were used for the inhibition studies, including ethanol, propanol, n-butanol, and isobutanol. It was expected that as the concentrations of alcohols increased, growth rates would decrease. This was true for the alcohols propanol, butanol and isobutanol. However, for ethanol experiments, as the concentration of ethanol increased, the growth rates also increased slightly (up to 4% ethanol). The experiment was then repeated with higher concentrations of ethanol. The repeated experiment showed that as the concentration of ethanol increased further, growth rates decreased. These results led to the directed evolution

experiments. Plots of biomass density (OD_{600}) and glucose concentration vs. time for the different alcohols are shown in figures 3.5 to 3.8.

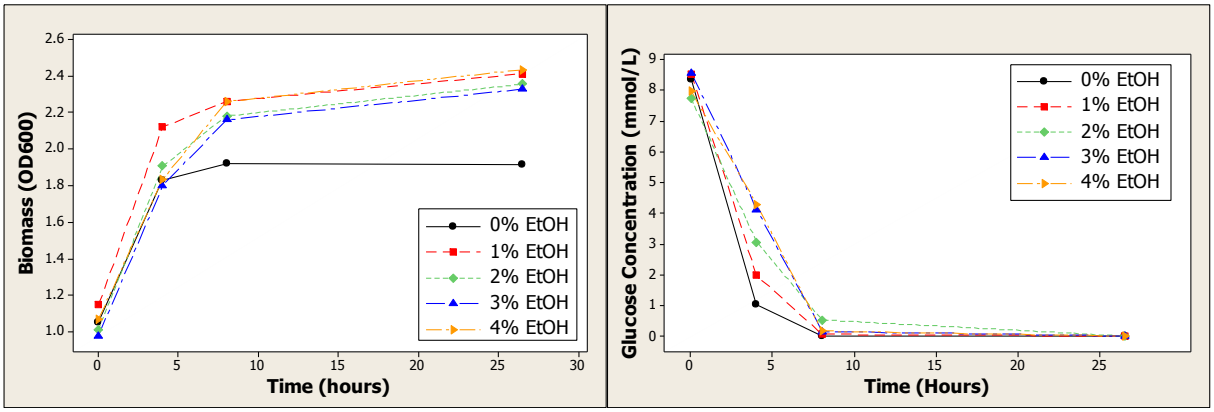

Figure 3.5: Biomass density and glucose consumption of *T. thermophilus* HB8 on added ethanol.

It was observed that propanol concentrations under 4% had a small effect on growth. Growth rates at higher propanol concentrations inhibited growth. However, inhibition effects were much more significant for butanol and isobutanol. For butanol, there was slight growth observed for a butanol concentration higher than 2%. Growth rates decreased dramatically from 2% to 3% of butanol. For isobutanol, almost no growth was observed for a concentration higher than 1% of isobutanol. From the investigated alcohols (ethanol, propanol, butanol, and isobutanol), isobutanol seemed to be the most toxic to *T. thermophilus*.

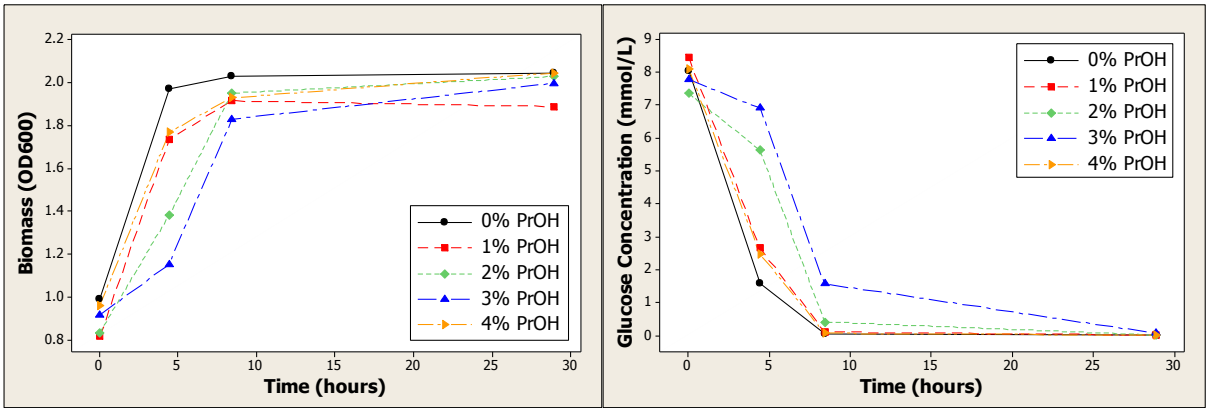

Figure 3.6: Figure Biomass density and glucose consumption of *T. thermophilus* HB8 on added propanol.

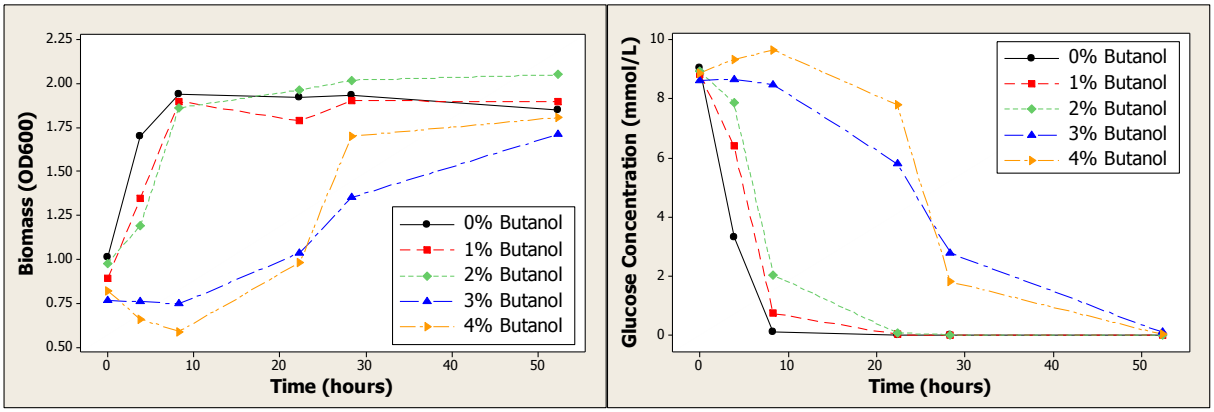

Figure 3.7: Figure Biomass density and glucose consumption of *T. thermophilus* HB8 on added butanol.

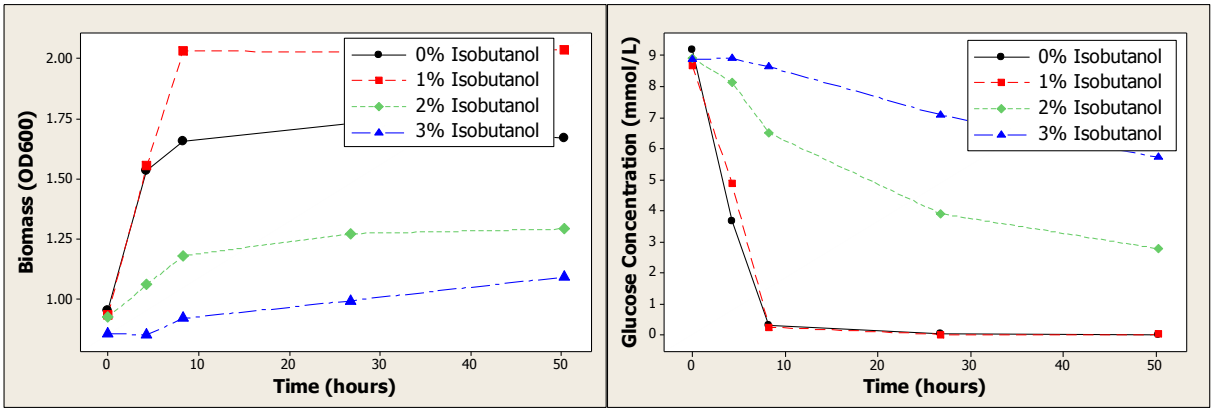

Figure 3.8: Biomass density and glucose consumption of *T. thermophilus* HB8 on added isobutanol.

3.5 Off-gas analysis results from labeled experiments

Off-gas measurements were taken by mass spectrometer during growth of cells to calculate gas composition including O_2, CO_2, and N_2. The CO_2 analysis provided the labeling as well as growth pattern of the cells. Growth rates were calculated from the amount of CO_2 released by the cells. Figure 3.9 shows the off-gas analysis data and a corresponding plot of growth rates calculated in figure 3.10.

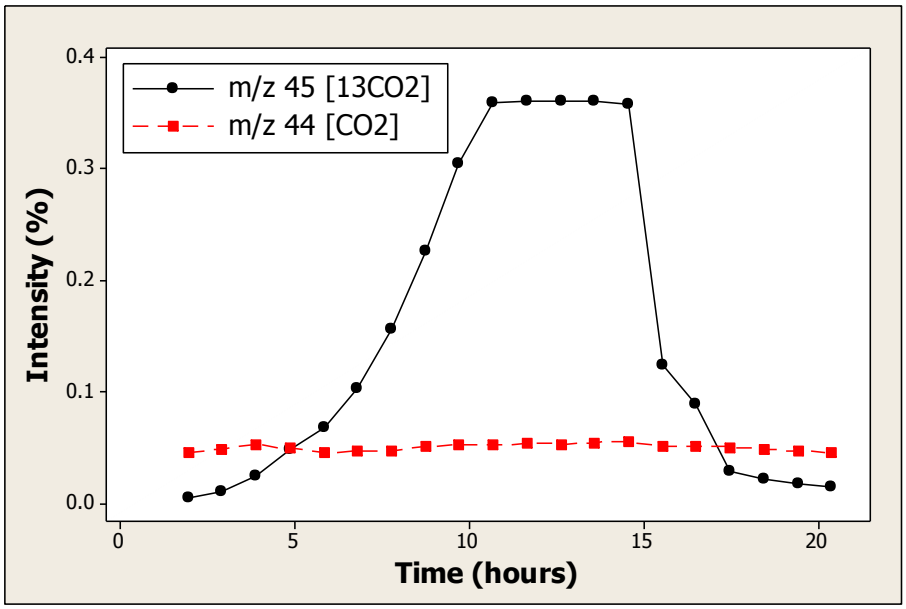

Figure 3.9: Growth profile of *T. thermophilus* from off-gas analysis supplemented with 4g/L of glucose, 0.5% Wolfe's minerals and 0.5% Wolfe's vitamins (no yeast extract).

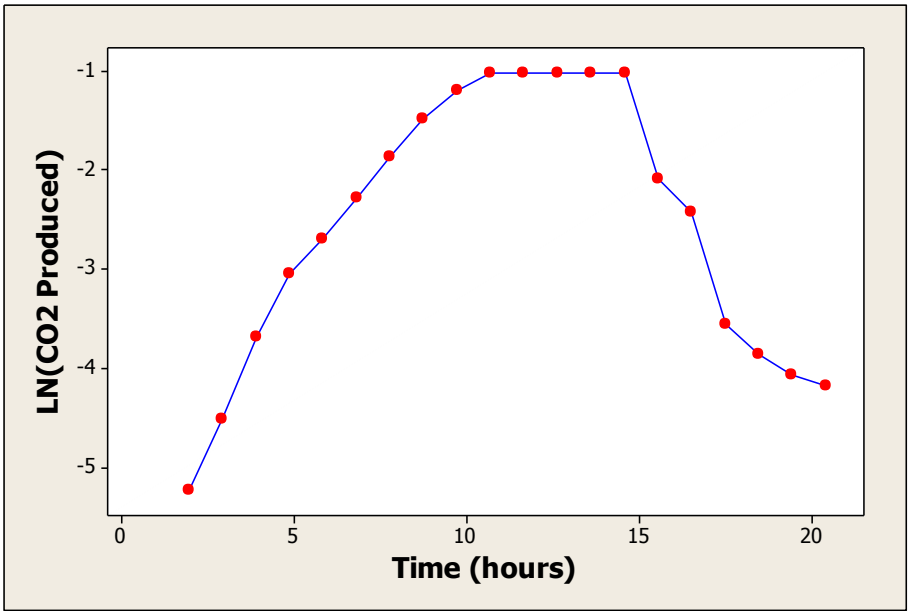

Figure 3.10: Growth rate of *T. thermophilus* supplemented with 4g/L of glucose, 0.5% Wolfe's minerals and 0.5% Wolfe's vitamins (no yeast extract).

3.6 Results from amino acid analysis

Mass isotopomer distributions (MIDs) were measured for amino acid fragments in experiments with [U-^{13}C]glucose as the isotopic-tracer. The chromatograms generated by the GC-MS were integrated and mass spectra obtained for each detectable amino acid fragment. MIDs were analyzed for the experiment with varying yeast extract amount, and the experiments with added ethanol. [U-^{13}C]Glucose tracer was used as the carbon source to determine the labeling of the amino acid fragments. The predicted average carbon labeling in [U-^{13}C]glucose experiments was 98% (i.e. 98 atom% ^{13}C-labeling of tracer, according to manufactures specification), if glucose was the sole carbon source and all labeling in the amino acid would originate from glucose. Figure 3.11 shows the average carbon labeling of 27 amino acid fragments for the varying yeast extract experiment. We observed that the average carbon labeling was lower than expected. Also, for increasing yeast extract

concentrations the average carbon labeling decreased, as can be seen from the plot below. The amino acid labeling was most likely diluted from the carbon sources contained in the yeast extract. For medium without yeast extract, the labeling could be diluted because of the initial inoculums, which was grown on unlabeled glucose. Additionally, there could also be some instrumental error from the mass spectrometer.

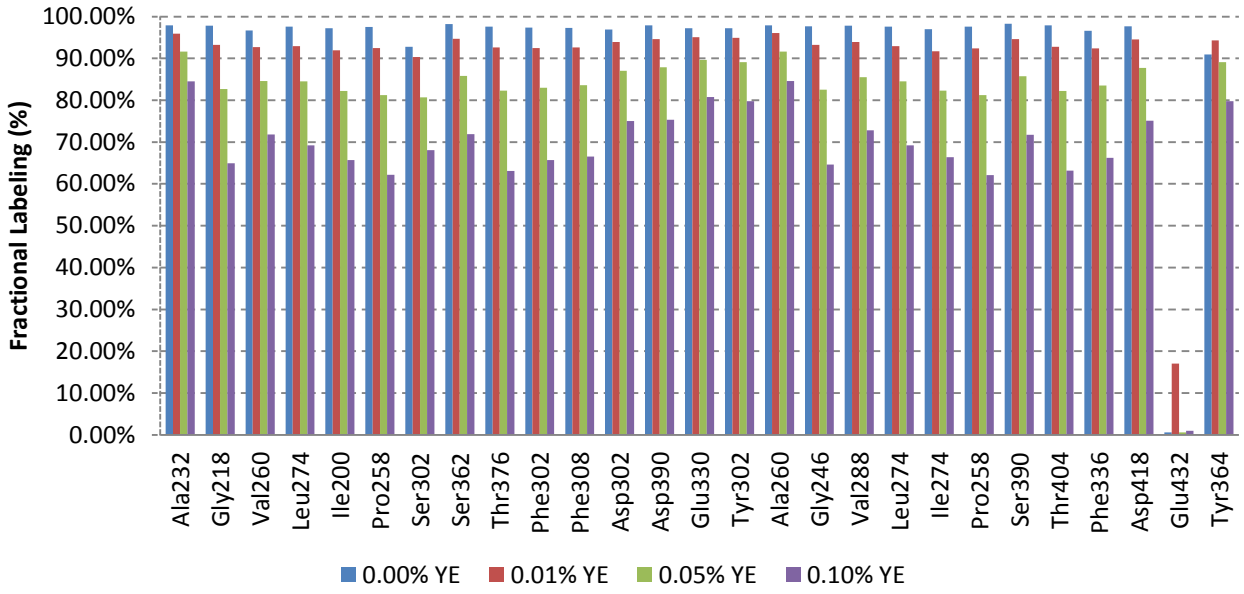

Figure 3.11: Fractional labeling of 27 amino acid fragments from HB8 cells grown on MRA2 medium with varying concentrations of yeast extract.

3.7 Directed evolution experiments

Next, to improve consistency of growth experiments, the HB8 cells were grown with the same medium and sub-cultured daily for several weeks until the cells reached consistent growth profile. The new cells were then used to repeat the glucose experiment in which different concentrations of glucose were used in the medium. Figure 3.11 and 3.12 show the biomass density and glucose concentration with the evolved JL3 and JL4 strains. Compared to

the starter strain HB8, the JL3 cells showed much better growth at higher glucose concentrations. The OD_{600} reached above 8.0 for 1.0% glucose compared to OD_{600} of about 1.1 for HB8 strain. Compared to HB8 strain, there was significant increase in glucose tolerance for the evolved cells.

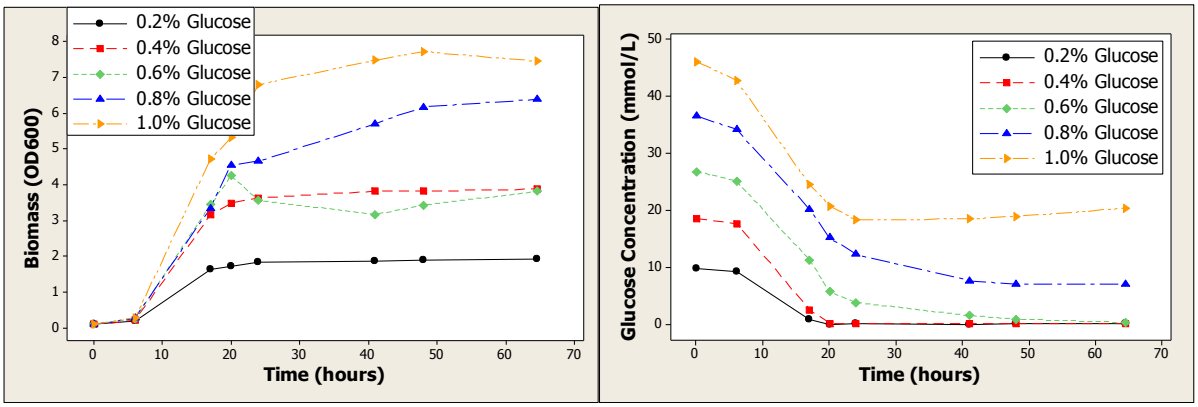

Figure 3.12: Biomass density and glucose consumption of *T. thermophilus* JL3.

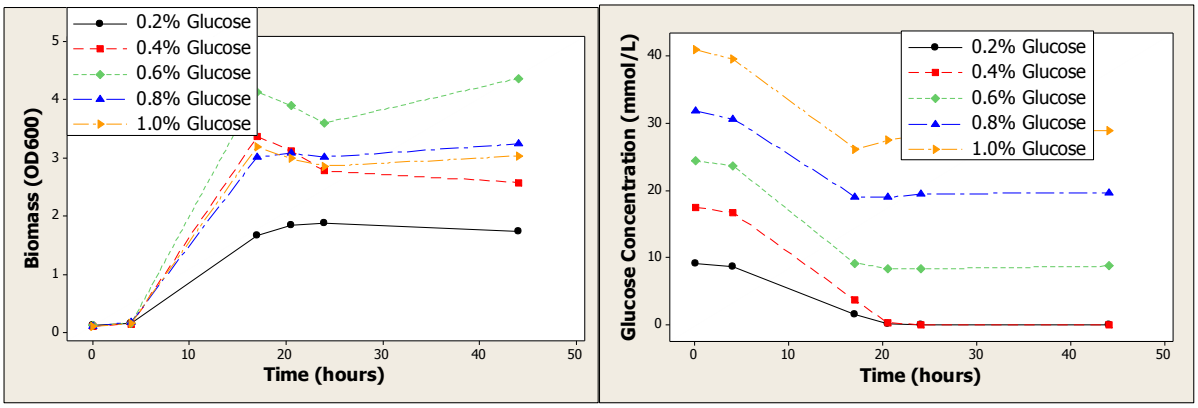

Figure 3.13: Biomass density and glucose consumption of *T. thermophilus* JL4.

Chapter 4

CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

In this work, the growth conditions for the thermophilic bacterium *T. thermophilus* were explored. This study shows that this bacterium can be used as a model organism for biofuels production.

In chapter 2, the experimental procedures were described for culturing this thermophilic bacterium. The goal of our experiments was to culture the bacterium in defined medium with glucose as the only carbon source. This was done in mini-bioreactors with a working volume of 10 mL. Later, the optimal growth temperature was determined for *T. thermophilus*. Finally, *T. thermophilus* underwent directed evolution to isolate new strains that were more tolerant to ethanol and high glucose concentrations. The experimental techniques for analyzing labeled amino acids were also described.

In chapter 3, we provide a detailed report of the results obtained from the batch cultures. During preliminary growth experiments, the defined growth medium was optimized to maximize the growth rate without the need to add yeast extract to the medium. After that, growth rates were measured under aerobic growth conditions for temperatures ranging between 50°C and 90°C with glucose as the sole carbon source. Based on the results, the optimal growth temperature was determined to be 80°C. The maximum growth rate for *T. thermophilus* HB8 was 0.28 1/hr, which corresponds to a doubling time of about 2.5 hours. Next, experiments were performed to determine the effects of medium supplements on growth rate. Supplement amounts were not changed because increasing the concentrations did not

have a major effect on growth rates. Next, growth conditions were examined for alcohol inhibition. Various alcohols were added to defined medium to study the effects on cell growth. At lower concentrations, the alcohols had insignificant effect on cell growth. However, at higher concentrations, cell growth was significantly inhibited. Different alcohols inhibited cell growth at different concentrations, with ethanol being the least toxic and isobutanol being the most toxic. Because we observed inconsistent growth for the HB8 strain in some experiments, directed evolution was employed to achieve more consistent growth profiles. Through these efforts, we obtained new, better growing strains of *T. thermophilus.* The new strains showed growth at higher glucose concentrations. The final defined medium was developed containing no yeast extract and glucose was used as the sole carbon source.

4.2 Recommendations for future work

4.2.1 Fed-batch experiments

We observed that the cells typically stopped growing when there was no more glucose, i.e. because the cells were carbon limited. Fed-batch fermentations could be performed in the future to determine the extent to which these cells can grow. In fed-batch fermentation, when glucose concentration in the bioreactors is low, glucose could be added so that the cells could continue growing. This would allow us to determine the growth pattern and maximum growth capacity of these cells.

4.2.2 Culturing under anaerobic conditions

In this project, the cells were grown aerobically. For effective production of biofuels using microorganisms, it is preferred to achieve anaerobic growth conditions. Using the defined medium developed for aerobic growth conditions and adding nitrate into culture

medium, anaerobic growth may be achieved, as was reported in the literature. The goal would be to optimize growth medium for high biomass density under anaerobic growth conditions.

4.2.3 Growth at high glucose concentrations

For the cells to be used industrially, growth rates need to be optimized for high glucose concentrations. For the evolved JL3 and JL4 strains, though they showed better growth under higher glucose concentrations, not all of the glucose was consumed. This may be due to waste product buildup inside the bioreactors. The goal of future experiments could be to determine the reason these cells stop growing and optimize the medium for growth at even higher glucose concentrations.

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