

## ABSTRACT

BERRY, RENEE NICOLE. Methanol Sensing and Control in a 2L Bioreactor for Design and Fabrication of a 30L Bioreactor Sensing and Control System. (Under the direction of Dr. Michael Flickinger).

Organisms capable of growing on methanol are of technological interest for single cell protein production, solvent degradation in aerobic thermophilic biotreatment processes, and fermentative production of amino acids (Arfman, 1992). *Bacillus methanolicus* and *Pichia pastoris* are two examples of methylotrophs that are versatile and robust in industrial and laboratory settings. Their ability to use methanol as a substrate and produce high product yields provides alternatives for microbial fermentation. Due to the toxicity of methanol at elevated concentrations the dissolved methanol concentration in the fermentation must be monitored. The pharmaceutical industry movement towards process analytical technology (PAT) requires real time data analysis by on-line sensing and control of process parameters. PAT will provide more efficient raw material utilization, higher product yield and reduced waste. In the case of methylotrophic organisms, sensors can be used to monitor the dissolved methanol concentration during the fermentation and feed the appropriate amount of methanol as needed by the organisms. The purpose of this research is to translate knowledge of dissolved methanol sensing from a 2L bioreactor model using *Bacillus methanolicus* to a 30L bioreactor for growth of *Pichia pastoris*. This included the fabrication and design of a steam sterilizable methanol probe for a 30L bioreactor using data collected from a 2L methanol probe and the implementation of new software and circuitry similar to the current 2L model.

Methanol Sensing and Control in a 2L Bioreactor for Design and Fabrication of a 30L  
Bioreactor Sensing and Control System

by  
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## **BIOGRAPHY**

After graduating from North Carolina State University in 2007 with a degree in Environmental Technology I worked as a Chemical Analyst at Novozymes for three years. There I discovered a passion for manufacturing and decided to further my education. Over the past two years I have focused my efforts on upstream process development and look forward to a rewarding career within the pharmaceutical industry.

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## LIST OF ABBREVIATIONS

AOX	Alcohol Oxidase
BTEC	Golden LEAF Biomanufacturing Training and Education Center
C <sub>1</sub>	One Carbon compound
HCD	High cell density
HCHO	Formaldehyde
MeOH	Methanol
MOS	Metal oxide sensor
MTYM	Media containing trace metals, yeast extract, and vitamins
OD, OD <sub>600</sub>	Optical Density, optical density at 600nm
PAT	Process analytical technology
RTV	Room temperature vulcanization
RuMP	Ribulose monophosphate cycle
WCW	Wet cell weight

## CHAPTER 1 INTRODUCTION

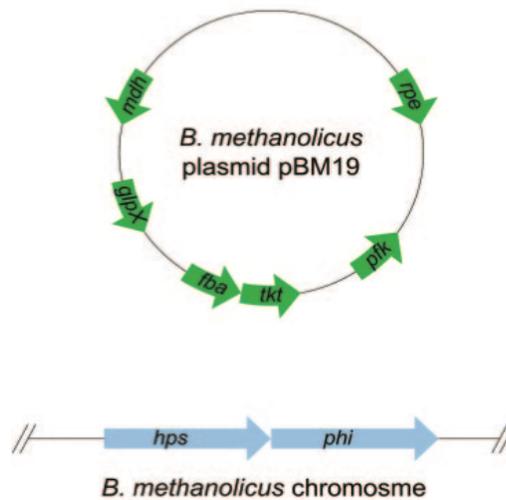
### 1.1 Importance of methylotrophic organisms and methanol

Methylotrophic organisms present an alternative way of producing biological products in microbial fermentations. Methylotrophic organisms can grow on reduced carbon compounds, such as methanol and methane (Vorholt, 2002). Some methylotrophs like *Bacillus methanolicus* and *Pichia pastoris* have the ability to reach very high cell densities (HCD) during fermentation and therefore increase product yields. These organisms are of interest for biotechnological applications, such as amino acid production, due to the relatively inexpensive feedstock as compared to other raw materials (Vorholt, 2002). Methanol is mostly produced from synthesis gas. Synthesis gas can be obtained from many different carbon containing compounds, such as coal, natural gas and oil. The worldwide availability of methanol and its independence from seasonal variations and weather conditions reveals an advantage to its use in industry (Schrader *et al.*, 2009).

### 1.2 General characteristics of *Bacillus methanolicus*

*Bacillus methanolicus* is a rod shaped, Gram-positive bacterium. It is studied extensively because of its tolerance to high temperatures and ability to grow on reduced carbon compounds. The optimal growth temperature for *B. methanolicus* is between 50 and 53°C and therefore reduces the cooling costs and risks of contamination during fermentation. The optimal pH range during growth is between 4 and 9 (Brautaset *et al.*, 2006). Methanol concentration must be continuously monitored during fermentation processes due to its toxicity at high concentrations from the formation of formaldehyde.

Metabolic engineering within *B. methanolicus* allows for higher tolerance (up to 1.5M) and higher consumption rates of methanol (Brautaset *et al.*, 2006). Methylo-trophy of *B. methanolicus* MGA3 is encoded on the circular plasmid pBM19. This plasmid contains six critical genes for the utilization of methanol via the ribulose monophosphate (RuMP) cycle as shown in Figure 1. Two genes that are also critical for methanol assimilation and tolerance are located on the bacterial chromosome (Brautaset *et al.*, 2006).



**Figure 1: Location of the RuMP genes in *B. methanolicus* plasmid pBM19 and bacterial chromosome (Brautaset, *et al.*, 2006)**

### 1.3 Pathways of methylotrophic bacteria for methanol assimilation

Methylotrophic organisms possess specialized enzymes that allow them to grow on reduced carbon substrates. Methanol is a commonly used one carbon ( $C_1$ ) substrate for many methylotrophic bacteria. A central feature of the metabolism of methylotrophic bacteria is that carbon flow proceeds via the central intermediate formaldehyde (HCHO), which is highly toxic due to its nonspecific reactivity with proteins and nucleic acids (Vorholt, 2002). Therefore, the metabolism of aerobic methylotrophic bacteria must proceed so that these binding reactions are prevented and formaldehyde consumption is efficient (Vorholt, 2002). The RuMP cycle in *B. methanolicus* assimilates formaldehyde into precursors for lipids, amino acids, and nucleic acids as shown in Figure 2 (Gutmacher, 2009).

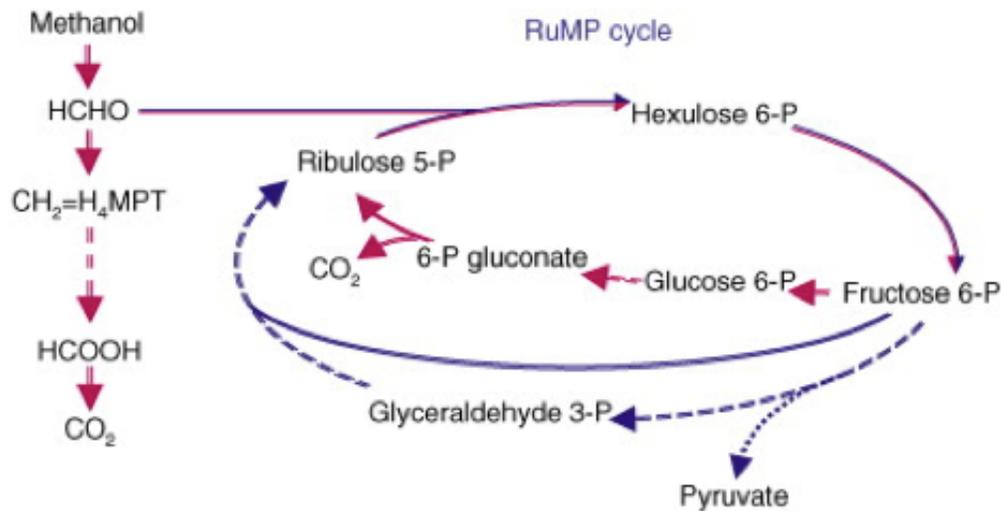


Figure 2: The Ribulose monophosphate (RuMP) cycle (Schrader *et al.*, 2009)

#### **1.4 General characteristics of *Pichia pastoris***

*Pichia pastoris* has become one of the most widely studied yeasts, since its development in the early 1970s, due to its useful and versatile systems for heterologous protein expression (Potvin *et al.*, 2012). *Pichia pastoris* has been used for the production of vaccines, coagulation inhibitors, fibrinolytic compounds, allergens, antibodies, protease inhibitors, hormones, cytokines, receptors, and ligands (Pingzuo *et al.*, 2007). This system is of particular industrial interest due to its powerful and tightly regulated methanol-inducible alcohol oxidase 1 promoter (AOX1). This system also has a high capacity for foreign protein secretion, ability to perform post-translational modifications, such as engineered glycosylation, and is capable of disulfide bond formation for expression of engineered antibodies (Potvin *et al.*, 2012).

#### **1.5 AOX1 promoter**

The methanol metabolic pathway utilizes a unique set of pathway enzymes. First, oxidation of methanol to formaldehyde must take place, which generates hydrogen peroxide by the enzyme alcohol oxidase (AOX). Hydrogen peroxide is toxic to the cell and so must be sequestered by the peroxisome. There are two genes in *P. pastoris* that code for the enzyme alcohol oxidase, AOX1 and AOX2. Expression of the AOX1 gene is tightly regulated and induced by methanol to high levels as shown in Figure 3 (Invitrogen Co., 2002). An important feature of *P. pastoris* expression vectors is the expression cassette. This includes the promoter AOX1. The design of this cassette will determine if the resulting product is mature and stable mRNA (Pingzuo *et al.*, 2007).

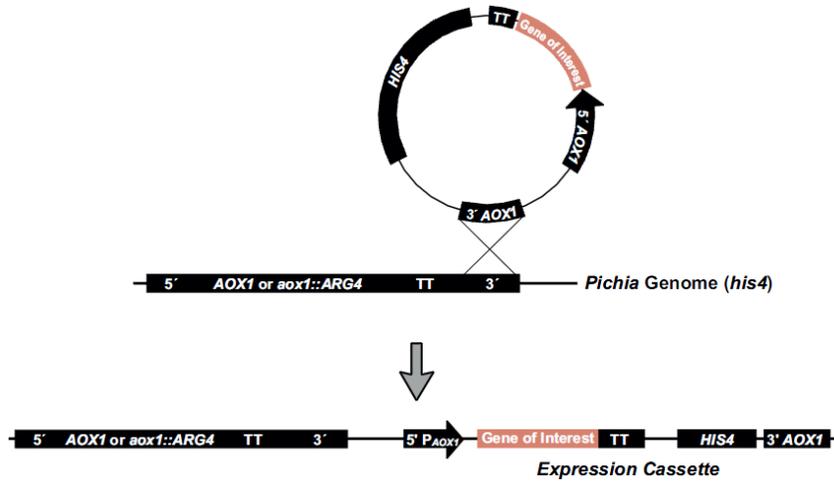


Figure 3: Insertion of plasmid 3' into AOX1 (Invitrogen Co., 2002)

## 1.6 Thesis Objectives

The objective of this research was to understand an existing 2L bioreactor system with methanol sensing and control capabilities. Through this understanding, design a new sensing and control system for a larger scale 30L bioreactor. This 30L system will utilize an on-line methanol probe, which was designed and fabricated based on small-scale studies. Many experiments included use of the 2L methanol probe and microbial growth curve data in order to scale up to the 30L system and implement a new sensing and control system based on these results. The 2L methanol probe is used only in small-scale studies and cannot be used within the 30L bioreactor because it is not long enough to have its silicone tubing submerged within the fermentation broth. This led to the need for a specifically designed and fabricated probe for the 30L bioreactor.

## CHAPTER 2 MATERIALS AND METHODS

### 2.1 Cultivation of *Bacillus methanolicus*

Laboratory work was performed under aseptic conditions in a vertical laminar flow biological safety cabinet in order to minimize contamination. *Bacillus methanolicus* is a Gram positive bacterium that is able to utilize methanol for growth. The wild type strain MGA3 was used. This thermotolerant strain is an obligate aerobe and requires biotin and vitamin B12 for growth. This strain is able to form endospores (Gutmacher, 2009).

#### 2.1.1 MTYM media

MTYM media was used for the growth of *B. methanolicus* in baffled 250mL Erlenmeyer flasks with milk filter disc closures. This medium is composed of a minimal salt solution, trace metals, vitamins and yeast extract and 150mM methanol. A complete list of medium ingredients is shown in Table 1. All three components, low salt solution, BFP trace metal solution, and the vitamin solution (1000x concentrate) are combined to form the MTYM media. The addition of a small amount of yeast extract and the concentrated vitamin solution ensure better growth of the organism.

**Table 1: Composition of MTYM media (adapted from Lindner, 2009)**

Ingredient	Concentration (g/L)	Description
NaH <sub>2</sub> PO <sub>4</sub> · H <sub>2</sub> O	1.5	Low Salt Solution Buffer
K <sub>2</sub> HPO <sub>4</sub>	4.1	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.1	
Yeast extract	0.25	
Na Citrate · H <sub>2</sub> O	26.64	BFP Trace Metal Solution
MgCl <sub>2</sub> · 6H <sub>2</sub> O	203.3	
MnCl <sub>2</sub> · 4H <sub>2</sub> O	19.08	
CaCl <sub>2</sub> · 2H <sub>2</sub> O	14.7	
FeSO <sub>4</sub> · 7H <sub>2</sub> O	11.12	
ZnCl <sub>2</sub>	0.27	
H <sub>3</sub> BO <sub>4</sub>	0.06	
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	0.1	
CoCl <sub>2</sub> · 6H <sub>2</sub> O	0.08	
CuCl <sub>2</sub> · 2H <sub>2</sub> O	0.05	
D-biotin (B <sub>7</sub> )	100	
Thiamine HCl	100	
Riboflavin (B <sub>2</sub> )	100	
Pyridoxine HCl (B <sub>6</sub> )	100	
Pantothenic acid (B <sub>5</sub> )	100	
Nicotinic acid (B <sub>3</sub> )	100	
p-aminobenzoic acid (B <sub>x</sub> )	20	
Folic acid (B <sub>9</sub> )	10	
Cyanocobalamin (B <sub>12</sub> )	10	
Lipoic acid	10	

### 2.1.2 *Bacillus methanolicus* shake flask studies

The *B. methanolicus* strain MGA3 was placed in 250mL baffled flasks covered with 2 milk filter discs and 30mL of MTYM media was added. The experiments were performed in triplicate. The flasks were placed in a shaker at 50°C at 300rpm overnight. Samples were taken every hour and the OD<sub>600</sub> was determined using a spectrophotometer in order to monitor growth.

### 2.1.3 Cryoconservation of *Bacillus methanolicus*

50mL of MTYM media were inoculated with 1mL of *B. methanolicus* strain MGA3 from an overnight starter culture. The growth of the inoculated culture was monitored until OD<sub>600</sub> readings reached 1.0. Glycerol was added to a final concentration of 10% and aliquots of 1mL were then flash frozen in liquid nitrogen and stored at -80°C.

## 2.2 Cultivation of *Pichia Pastoris* and media composition

Laboratory work was performed under aseptic conditions in a vertical laminar flow biological safety cabinet at BTEC. *Pichia pastoris* BEC7 was grown in chemically defined media using a recipe from Invitrogen and in YPD media. The most common rich medium for cultivation is YPD (1% yeast extract, 2% peptone, 2% dextrose). Growth of *P. pastoris* on methanol requires that the dextrose be replaced with methanol to 0.5%(v/v) in YPD. *Pichia pastoris* has a generation time of nearly 90 minutes in YPD and a generation time of nearly 3 hours in defined medium.

### 2.2.1 *Pichia pastoris* shake flask studies

The *Pichia pastoris* BEC7 strain was inoculated in duplicate 250mL baffled Erlenmeyer flasks with 30mL of YPD media and milk filter disc closures. The flasks were placed in a shaker at 30°C at 300rpm overnight. Samples were taken the next day and OD<sub>600</sub> was measured using a spectrophotometer in order to monitor growth. These starter cultures were used to inoculate two 250mL Erlenmeyer flasks with either 30mL of YPD or chemically defined media. Samples were taken every 2 hours to monitor growth by OD<sub>600</sub> measurements.

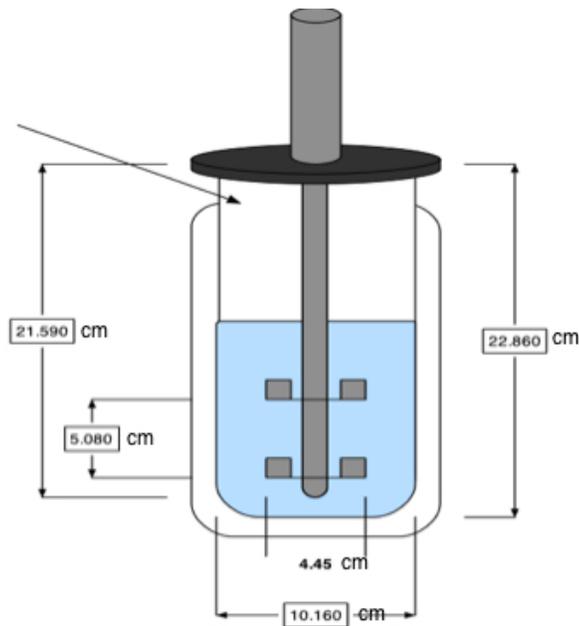
### 2.3 2L Bioreactor System

Studies were carried out in a 2L total volume Applikon<sup>®</sup> Model P140 bioreactor. The operating volume was 1L. The dimensions are shown in Figure 4 and Table 2 for the 2L bioreactor. Figure 5 and Table 2 are the dimensions of the 30L bioreactor. The geometry of the two systems must be considered for scale-up and mixing studies. The 2L methanol probe and the 30L methanol probe were calibrated before experiments were performed.

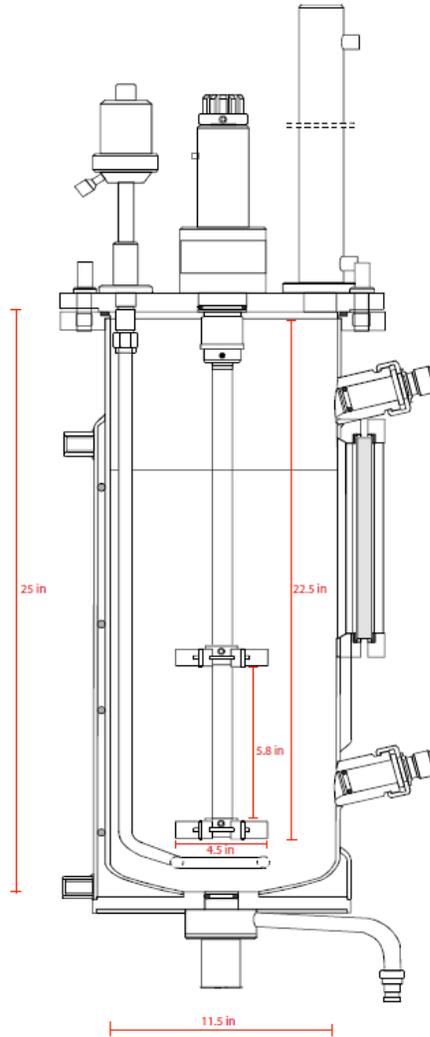
**Table 2: Dimensions for 2L bioreactor and 30 L bioreactor**

2L Measurement	Result	30L Measurement	Result
Height/ diameter ratio	2:2:1	Height/ diameter ratio	2:2:1
Total Volume	2 L	Total Volume	42 L
Working Volume	1 L	Working Volume	30 L
Vessel internal diameter	10.2 cm	Vessel internal diameter	29.2 cm
Vessel height	22.9 cm	Vessel height	63.5 cm
Impellers	2	Impellers	2
Impeller diameter	4.5 cm	Impeller diameter	11.4 cm
Distance between impeller	5.1 cm	Distance between impeller	14.7 cm

The 2L bioreactor is glass with a surrounding water jacket for heating and cooling. It is equipped with two, six blade Rushton turbines propelled by a DC motor Applikon® P140. The Rushton turbine is an impeller with six vertical disks mounted on a flat disk and used for gas dispersion. During experiments the agitation rate was set at 700 rpm. The temperature was monitored by a thermocouple and maintained at ambient conditions for comparable scale up studies in the 30L bioreactor due to problems with the heating unit on the current 30 L Sartorius unit. A new heat exchanger was ordered to solve the problem.



**Figure 4: 2L Applikon® bioreactor**



**Figure 5: 30L Sartorius BIOSTAT® bioreactor**

The current 2L system is shown in Figure 6. The system is capable of monitoring process parameters such as, temperature, pH, dissolved methanol, and carbon dioxide exhaust. The parameters are monitored via system design software that can be programmed to measure and control systems using LabVIEW™ software.

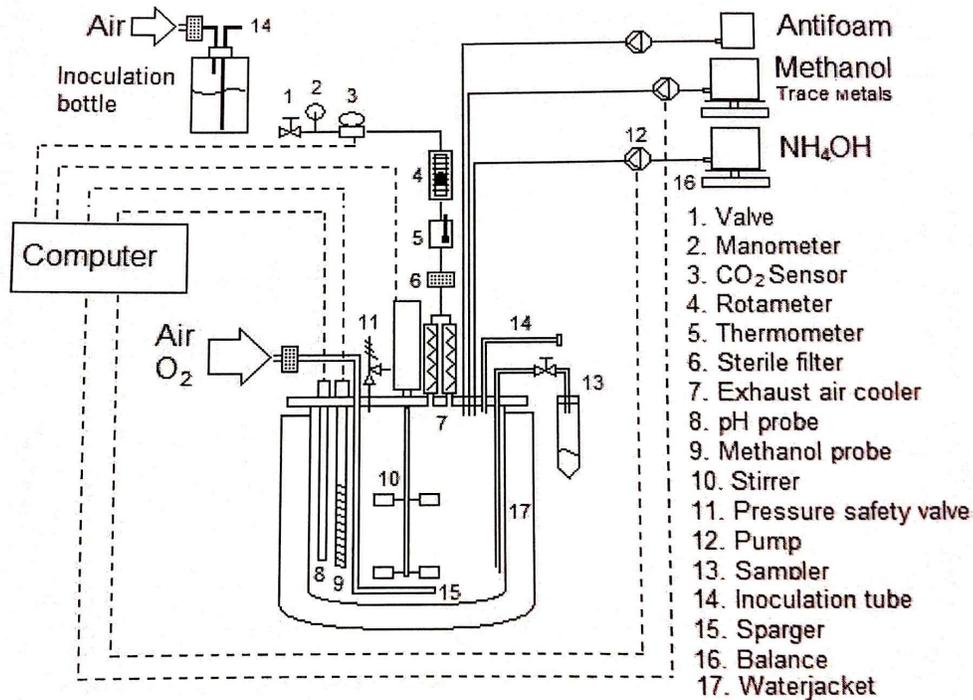


Figure 6: Current 2L model (Gutmacher, 2009)

## 2.4 Methanol Probe for 2L bioreactor

A method termed “the silicone tubing method” was originally used for measuring dissolved oxygen, and later used to determine methanol concentrations. The method works by allowing methanol within the fermentation medium to diffuse through a silicon tube. The silicon tube must be fully submerged in the fermentation medium and is wrapped around a support to form a probe. The dissolved methanol that diffuses through the silicone tubing wall is swept to a hydrocarbon analyzer by an inert gas, such as dry nitrogen that is flowing within the silicon tube lumen.

Figure 7(a) shows the dimensions of the methanol probe used for the 2L bioreactor with the silicone tubing wrapping around the lower portion of the probe. Figure 7(b) shows the inner working of the probe and how dry nitrogen is introduced at the inlet of the probe and carried within the silicone tubing inside a hollow glass tube; the silicone tubing wraps around the glass tubing. The tubing then reenters the hollow glass tube and out through the top of the probe to the hydrocarbon sensor. The silicone tubing that is wrapped around the outside of the glass tube has a total surface area of 35.5 cm<sup>2</sup> as shown in Table 3.

The concentration of hydrocarbon exiting the silicone tube ( $c_t$ ) can be determined using Equation (1) and by knowing the bulk concentration ( $c^*$ ), diffusivity ( $D$ ), length of tubing ( $L$ ), flow rate of carrier gas ( $Q$ ), partition coefficient ( $H$ ), and inside ( $a$ ) and outside ( $b$ ) radius of silicone tubing (Austin *et al.*, 1992). This equation assumes that liquid film resistance is negligible, the carrier gas flow was represented by plug flow, and the methanol concentrations at the outer and inner surface were in equilibrium.

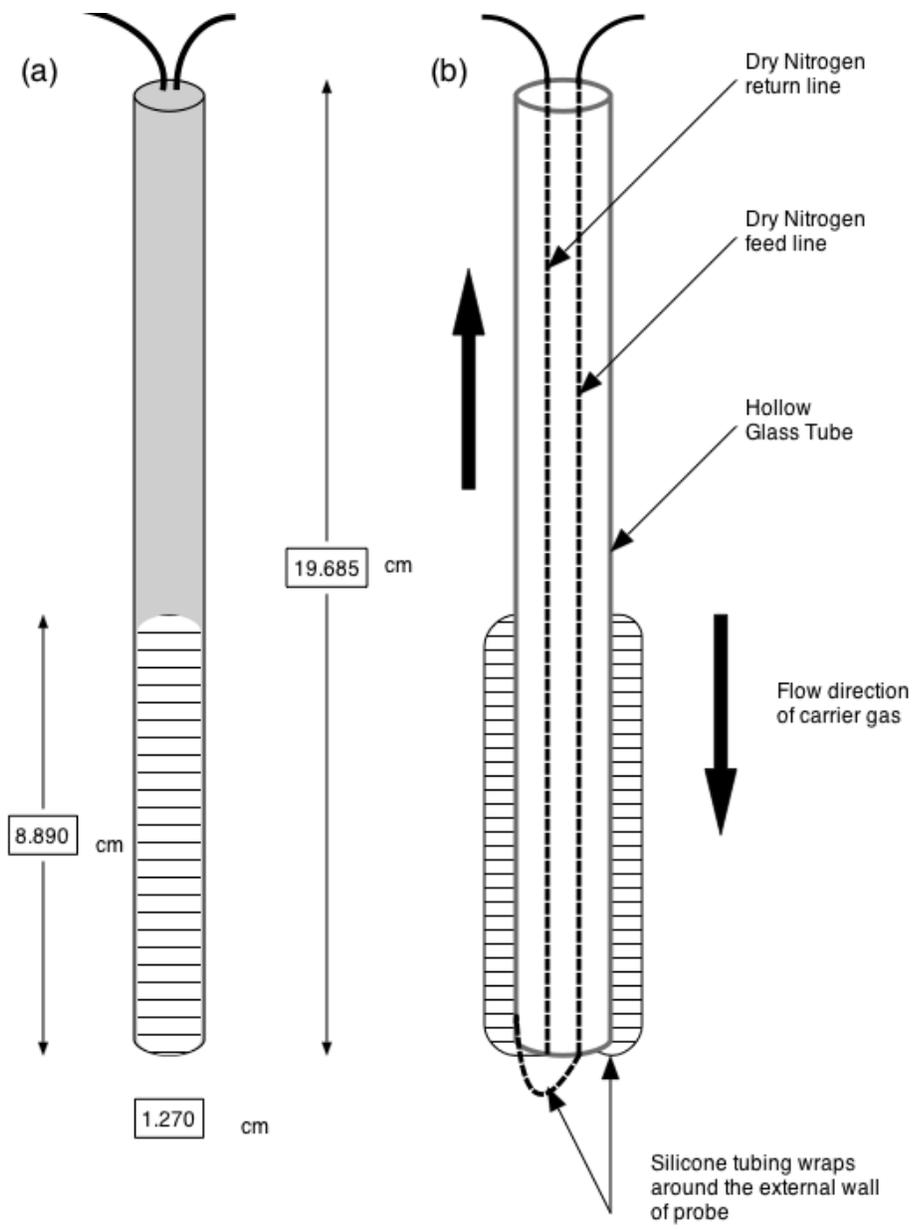
$$c_t = \left( 1 - \left[ \exp \left( -2\pi \frac{DHL}{Q \ln \left( \frac{a}{b} \right)} \right) \right] \right) c^* \quad (1)$$

In order to determine this bulk concentration by analytical methods, samples from the exit stream can be analyzed by a gas chromatogram, but that is expensive and has a time delay. A flame ionization detector may also be used to measure methanol gas composition, but is very costly and moisture accumulation within the tubing can be problematic.

The implementation of an inexpensive metal oxide sensor (MOS) can be used with an on-line methanol probe to determine methanol concentration in real time. Utilizing principles of membrane separation and semiconductor gas sensor technology, a hydrocarbon sensor, methanol probe, and data analysis software can be used to follow methanol concentrations on-line (Austin *et al.*,1992). This method is less expensive than other analytical methods and it allows for analysis of real time dissolved methanol levels in the fermentation.

**Table 3: Silicone tubing dimensions and methanol data**

Silicone tube to sensor	78.7	cm
Surface area of silicone tubing	35.4	cm <sup>2</sup>
MeOH MW	32.0	g mol <sup>-1</sup>
MeOH density	0.792	g/mL
Tubing outer diameter	0.12	cm
Tubing inner diameter	0.06	cm
Tubing wall thickness	0.06	cm

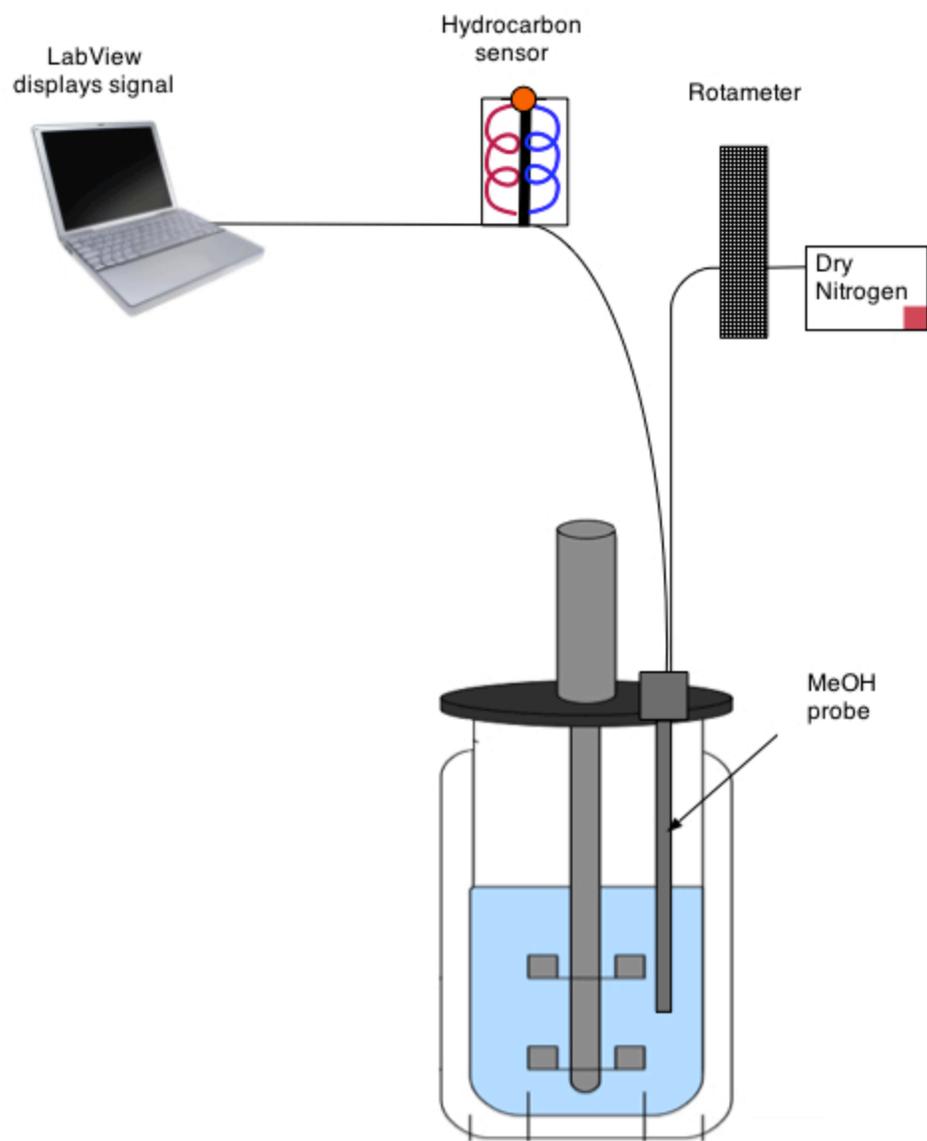


**Figure 7: (a) 2L MeOH probe dimensions (b) Internal silicon tubing and carrier gas transport workings for the 2L MeOH probe**

## 2.4 Methanol sensing and control system in the 2L bioreactor

The dissolved methanol concentration within the bioreactor can be monitored utilizing a methanol probe, hydrocarbon sensor, and LabVIEW™ software as shown in Figure 8. The software LabVIEW™ monitors parameters such as, temperature, pH, dissolved oxygen, dissolved methanol, exhaust gas volume, oxygen gas flow and agitation speed. The focus for these experiments was on the dissolved methanol measurements.

The hydrocarbon sensor by Figaro TGS-822 is a tin dioxide ( $\text{SnO}_2$ ) semi-conductor that shows an increase in conductivity when a hydrocarbon such as methanol is present and a low conductivity reading to air. The circuit can convert this measured change in conductivity to a signal that can be correlated to methanol concentration by performing a probe calibration. Figure 8 shows how dry nitrogen from a cylinder is measured by a rotameter to maintain a steady flow rate. The dry nitrogen is a carrier gas within the silicone tubing that is shown entering the probe within the bioreactor, wrapping around the probe in order to be in contact with the media. The silicone tubing then exits the probe and is connected to the hydrocarbon sensor. The hydrocarbon sensor measures conductivity changes in the carrier gas due to the presence of methanol. The signal is converted into a concentration reading within LabVIEW™.



**Figure 8: MeOH sensing and control in the 2L bioreactor**

## CHAPTER 3 RESULTS AND DISCUSSION

### 3.1 Shake flask growth of *Bacillus methanolicus*

Shake flask experiments were performed to determine the growth rate of *B. methanolicus* in order to design experiments for determining methanol uptake rate and future scale up studies. It is important to understand the mass balance of *B. methanolicus* by measuring inputs and outputs. The growth rate will help to determine the methanol uptake rate and what amount of methanol is needed for the growing organism as an input.

An overview of the mass balance of *Bacillus methanolicus* is shown in Figure 9 and includes the inputs and outputs of the organism.

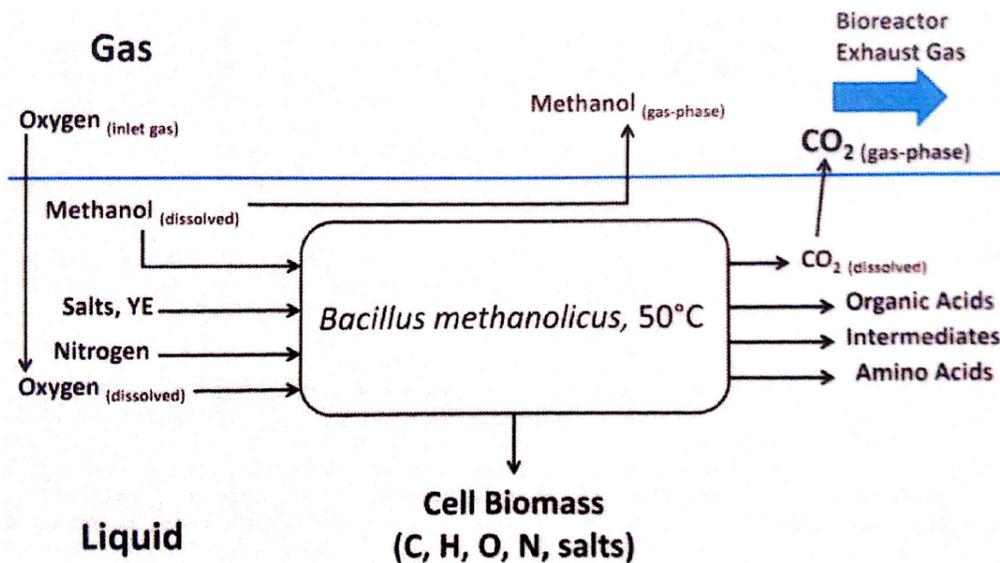
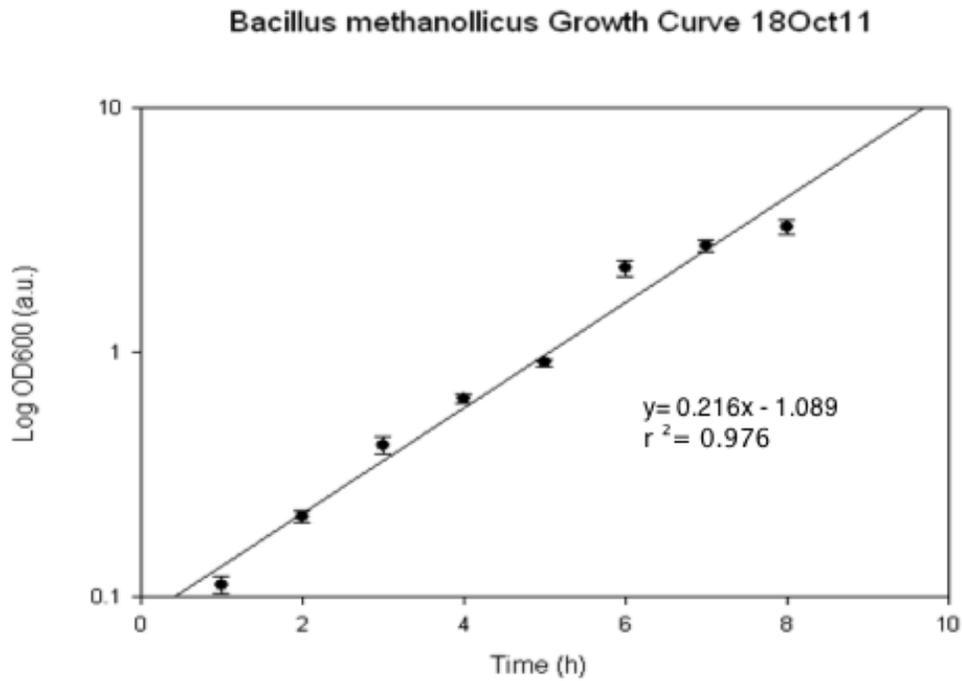


Figure 9: Mass balance of *Bacillus methanolicus* (Lindner, 2009)

Figure 10 shows the measured growth as the natural logarithm of the optical density as a function of time. The maximum specific growth rate ( $\mu_{\max}$ ) over a four-hour period for this experiment was  $0.23 \text{ h}^{-1}$ . The maximum growth rate for *B. methanolicus* is  $0.475 \text{ h}^{-1}$  based on literature review. Possible reasons that the organism did not reach the maximum growth rate are lack of oxygen due to increased time out of the shaker and temperature controlled area by sampler.



**Figure 10: The growth of *B. methanolicus* in shaker flasks at  $50^{\circ}\text{C}$  in MTYM media**

The methanol uptake rate was determined by Equation (2) to be 118.40g MeOH Lh with the maximum growth rate defined by literature review.

$$\mathbf{MeOH\ UR} = \frac{\mu_{max}X}{Y_{x/MeOH}} \quad (2)$$

The cell yield is assumed to be 165 gWCW/L and the yield per gram of methanol is 0.662 gWCW/gMeOH. These values were used to calculate the maximum methanol uptake rate.

### **3.2 Shake flask growth of *Pichia pastoris***

Culture media composition and components such as dissolved oxygen and dissolved methanol concentration all affect the product yield from *Pichia pastoris* expression systems. Methanol uptake rate by the organism is important to understand because it can be used to maintain the optimal methanol concentration within the media. After performing shake flask studies and determining the growth rate of *Pichia pastoris* BEC7 the MeOH UR was calculated using (2). Figure 11 shows the mass balance of inputs and outputs for *P. pastoris*. The growth rate helps to determine the MeOH UR and oxygen uptake rate by the organism. These are important parameters to know when predicting maximum cell yield and recombinant protein production.

The graph in Figure 12 shows the average of the measured growth as the natural logarithm of the optical density as a function of time. The growth curve was measured during a 12-hour period.

The methanol uptake rate for *Pichia pastoris* was determined to be 52.9 gMeOH Lh in YPD media . The cell yield per gram of methanol is 1.19 gWCW/gMeOH while the cell yield (X) is 450 gWCW/L.

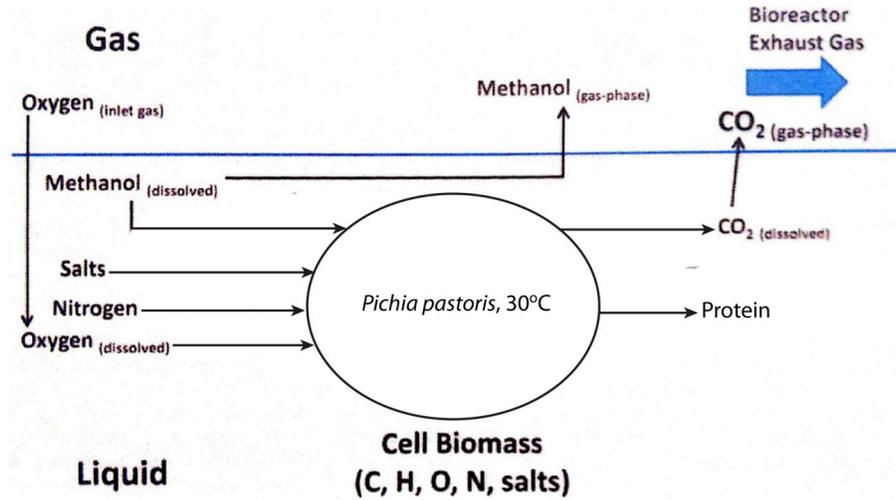
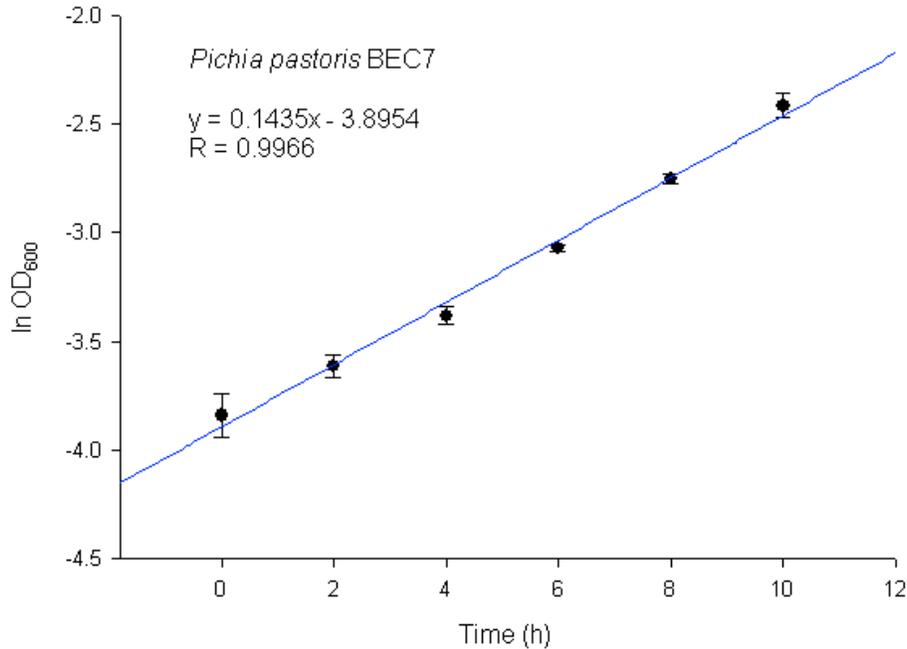


Figure 11: Mass balance of *Pichia pastoris* (Lindner, 2009)



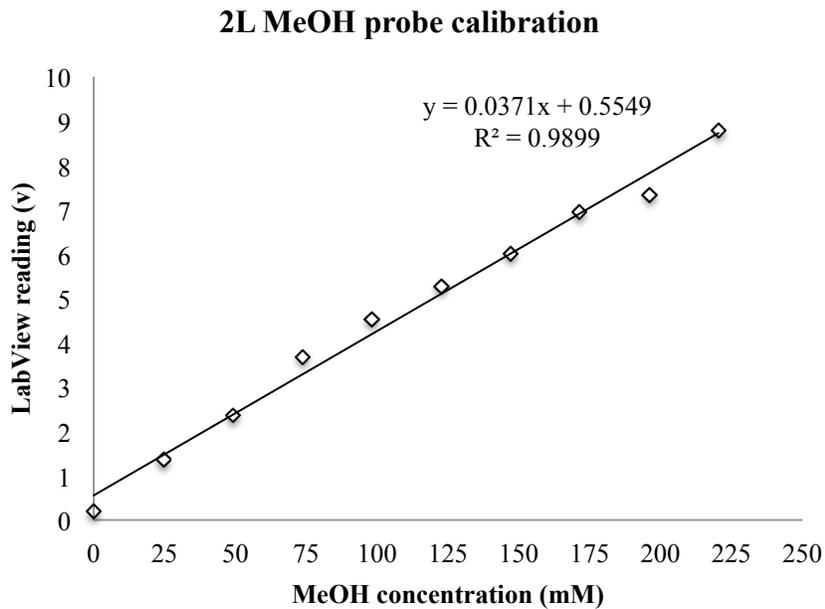
**Figure 12: Growth curve of *P. pastoris* BEC7 in shaker flasks containing YPD media**

### 3.3 Methanol Probe Experiments

#### 3.3.1 2L Methanol Probe Calibration for 2L bioreactor

The methanol probe was calibrated by filling the reactor with 1L of deionized water. The agitation speed was set to 700 rpm and the temperature was 23.5°C. Since the 30L bioreactor probe calibration was completed at ambient temperature due to an inoperable temperature control system, the 2L system used the same parameters for consistency. The outlet pressure on the nitrogen cylinder was 10 psi while the calibration was performed. The rotameter was set at 50 and the dry nitrogen flow rate was 103.5 mL/min during calibration.

The methanol probe was calibrated in a range of 0 to 220.4mM methanol by adding 1mL of methanol every 10 minutes with a micropipette and awaiting stabilization in LabView™. The methanol was added directly into an opening on the headplate of the 2L bioreactor. Figure 13 shows the calibration curve with a correlation of which 0.9899 and is acceptable for the standard curve. The probe should be calibrated with the actual fermentation medium to be used during experiments and after steam sterilization to ensure that the signal is correlated to the correct methanol concentration.

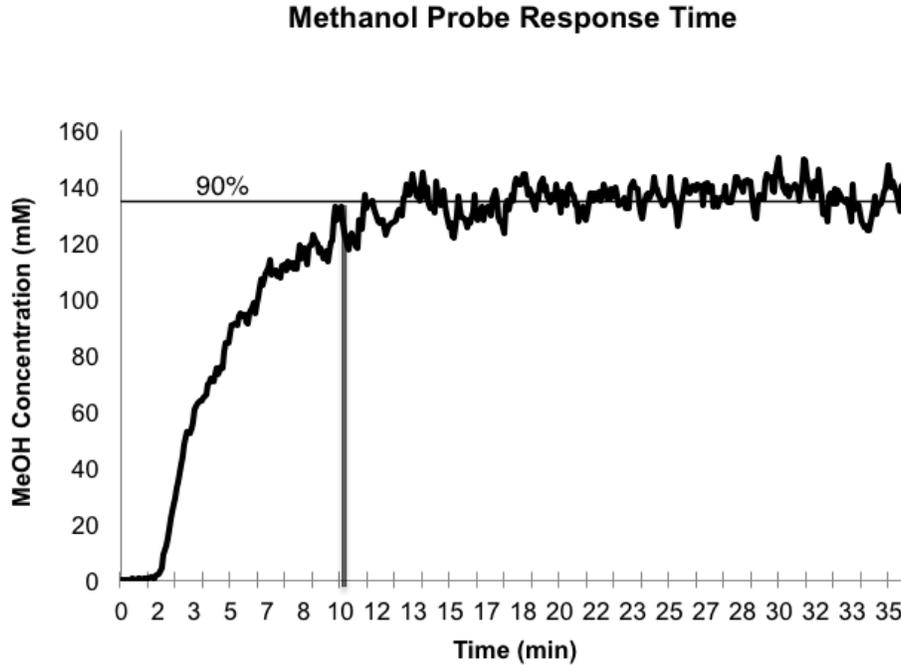


**Figure 13: Calibration of the 2L MEOH probe**

### 3.3.2 Response Time of 2L Methanol Probe

To determine the response time of the methanol probe, methanol was added to the bioreactor to a final concentration of 150mM and the time for the signal to reach 90% of the known concentration was recorded. The agitation speed was set at 700 rpm and the temperature was 23.5°C. The nitrogen flow rate was 103.5mL/min. Figure 14 shows the response time to be 11 minutes for the sensor to detect the change in concentration of methanol at 90% of 150mM methanol (135mM). Many factors affect the response time of the methanol probe as shown in Equation (3). These include the amount of silicone tube area exposed (A), the permeability coefficient of the silicone tubing (P), the tubing wall thickness (b), and the MeOH concentration. Additional factors are the time to reach the steady state between the liquid phase and the sensor internal gas phase and the membrane transfer properties. The temperature, agitation rate, pH, path length of tubing to sensor, and the carrier gas flow rate can influence these factors.

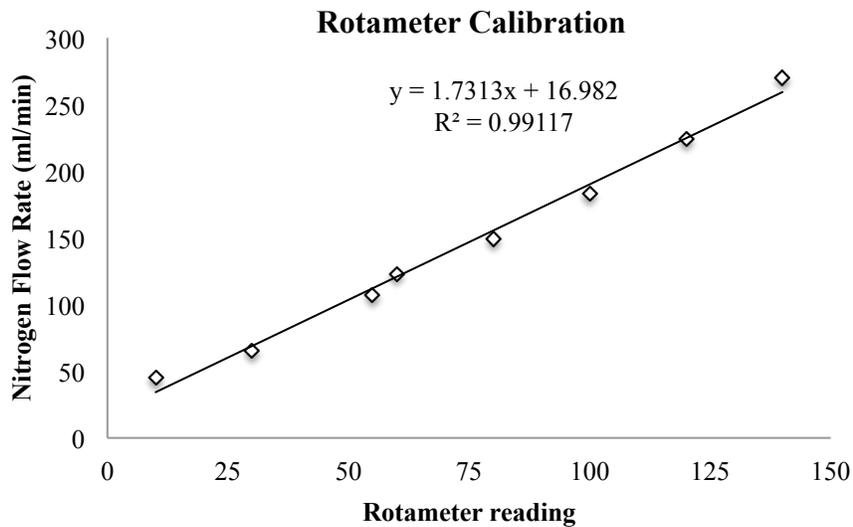
$$\Delta \text{ Output signal} \propto A \left[ \frac{P}{b} \right] [\text{MeOH}] \quad (3)$$



**Figure 14: Probe response time determined for a signal to detect 90% of the predicted methanol concentration**

### 3.4 Carrier gas rotameter calibration for 2L bioreactor

The rotameter used to measure the flow rate of dry nitrogen gas entering into the methanol probe was calibrated using the water displacement method. The rotameter displays readings from 0 to 150. The rotameter was set to eight different values and then the water displacement was measured for 60 seconds at each setting. The results are shown in Figure 15. The  $R^2$  value was 0.9917 and revealed a strong linear correlation. During the 2L probe experiments the rotameter was set at 50. This value correlates to a flow rate of 103.5mL/min of dry nitrogen carrier gas into the silicon tubing.



**Figure 15: Calibration curve for nitrogen gas rotameter**

### 3.5 Transfer Studies to 30L Bioreactor

#### 3.5.1 30L Methanol Probe Design

The probe design for the 30L bioreactor had many considerations, including shape, size, placement, material, and liquid level of the bioreactor. The probe design is pictured in Figure 16 and was fabricated with stainless steel SS316. One early determination to be made was the probe placement. Originally, the probe was designed for the probe belt; however future fermentations would need probes for OD, dissolved oxygen, temperature and pH, located in the four openings of the probe belt. Therefore it was decided to insert the probe through the headplate, making the probe length 650mm, but the design was effective as long as the silicon tubing was submerged within the bioreactor at minimum liquid level of 9 L.

The probe had to fit into an existing opening in the headplate and the diameter had to be less than 14mm. The silicone tubing was sealed to the stainless steel tubing ends with room temperature vulcanization (RTV) silicone adhesive and cured over a 24-hour period. Two 1/16<sup>th</sup> inch ball valves were placed on the inlet and exhaust lines for the nitrogen to flow in and out of the sensor. Once application of the RTV and valves was complete, air tests were performed under water to ensure that no leaks were evident in the tubing or between the tubing fit to the 1/16<sup>th</sup> stainless steel tubes. Figure 17 is an image of the final 30L probe. Two filters are connected to the stainless steel SS 316 tube to ensure that the nitrogen gas is completely dry and free of particulates. The silicone tubing is shown wrapped around the bottom of the probe. The amount of tubing was determined based on the minimum working volume of the 30L bioreactor, which is 9 L. This design allows the silicone tubing to be submerged.

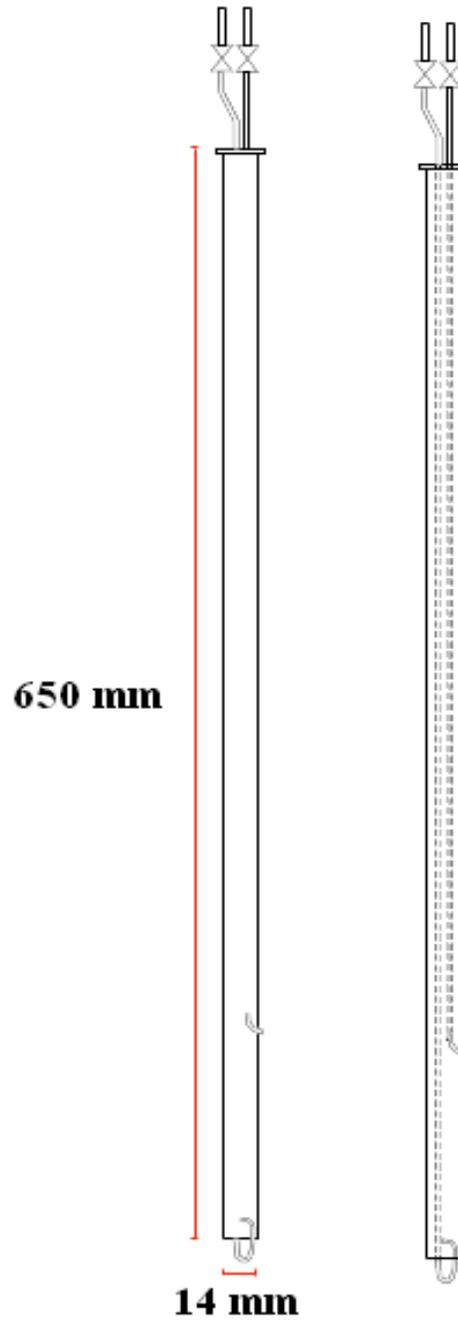
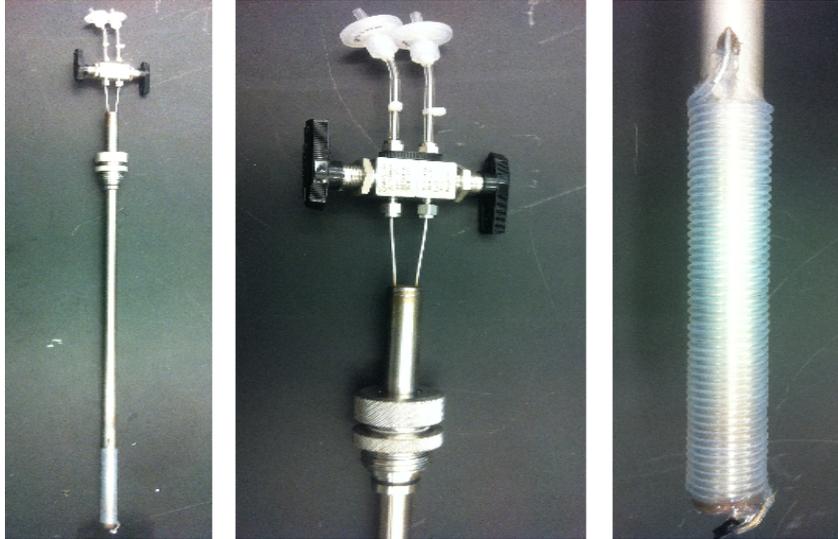


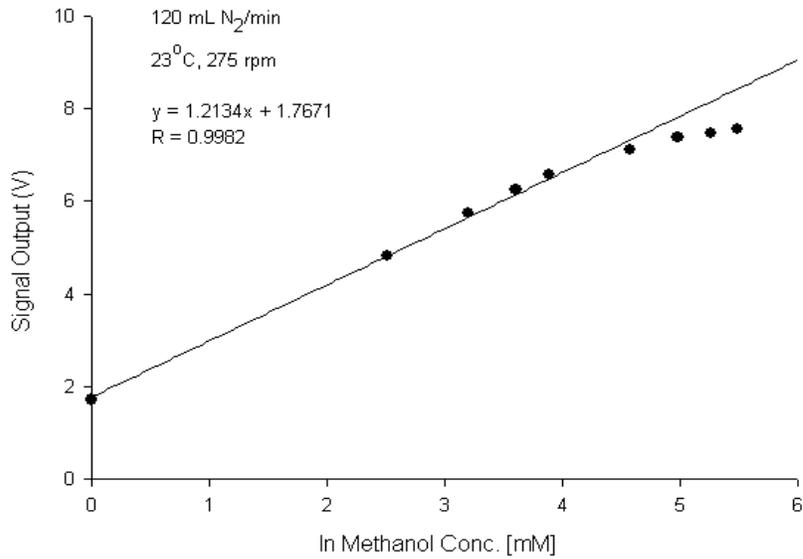
Figure 16: Final 30L methanol probe design



**Figure 17: Image of the 30 L methanol probe with ball valves and silicone tubing (Cueva, 2012)**

### 3.5.2 Methanol probe calibration 30L

The 30L probe was calibrated with a correlation coefficient of 0.9982. Additional studies will be needed to optimize the calibration, however the hydrocarbon sensor is relaying a signal to LabVIEW™ as shown in Figure 18.



**Figure 18: Calibration of 30L MeOH probe (Cueva, 2012)**

### 3.5.2 Proposed 30L system design

A proposed design for the 30L system is depicted in Figure 19. The 30L methanol probe is currently generating a signal in LabView™. Further focus should be placed on implementation of exhaust gas analysis utilizing BluSens™ technology and the mass flow meters already installed.

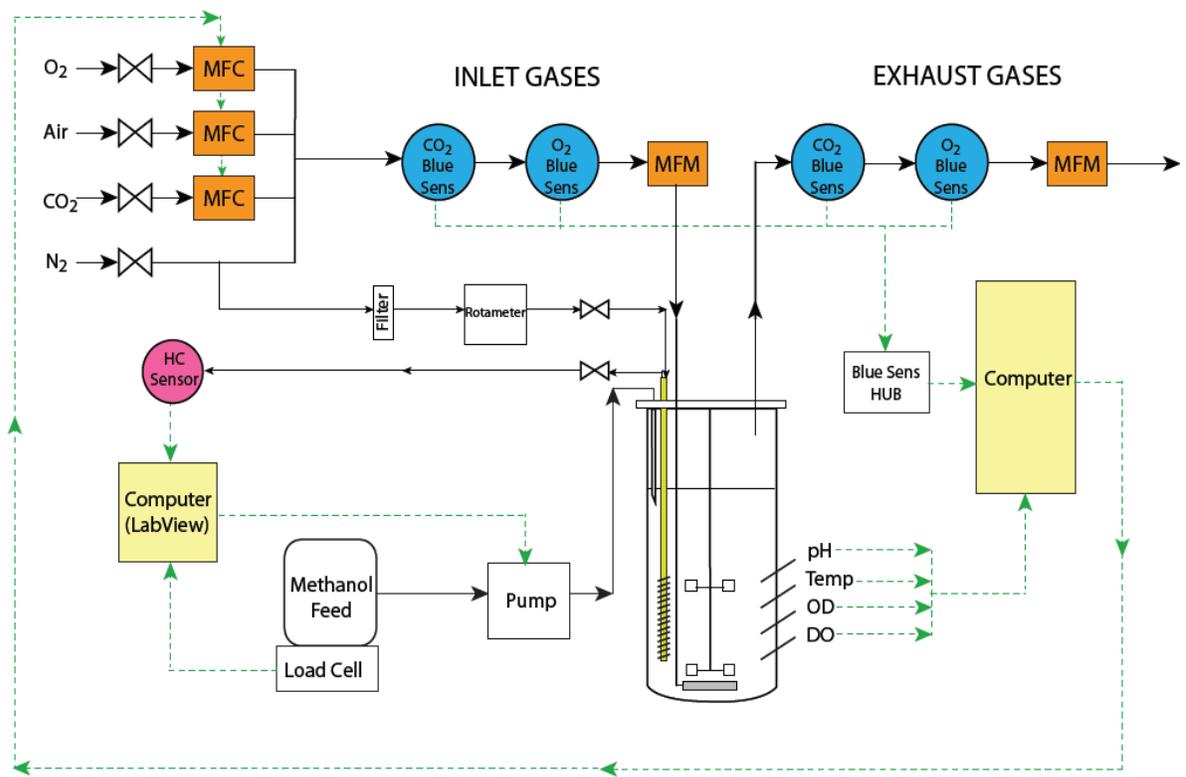


Figure 19: Proposed 30 L system (Cueva, 2012)

## CHAPTER 4 CONCLUSION

### 4.1 Conclusion

The ability to use an inexpensive hydrocarbon sensing and inexpensive methanol probe in order to determine on line dissolved methanol concentration is a beneficial way to monitor fermentation parameters in real time. The 2L bioreactor system is functional and can be used for small-scale studies. The probe response time was calibrated and the dissolved methanol concentration within the bioreactor does correlate with the voltage reading from the hydrocarbon sensor. Furthermore, additional equipment within the 2L model system is now calibrated. The growth curve data from *Pichia pastoris* and *Bacillus methanolicus* can be used to establish larger scale studies. These experiments allowed for a great learning opportunity.

The knowledge of the 2L system methanol probe was critical in designing the 30L methanol probe that is currently installed at BTEC lab 218. The design consideration and sensing and control system were implemented based on the previous system and the 30L system methanol probe did generate a signal within LabView™.

This experience has provided me with many new technical abilities, including electrical engineering concepts, improved bioreactor functioning, and increased knowledge of scale up studies.

## 4.2 Future Work

The next phase of the project will be exciting and rewarding. Now that the sensing system is constructed in the 30L bioreactor system it can begin to be utilized and optimized for *P. pastoris*. Additional tests on the 30L methanol probe are needed to determine the optimum nitrogen flow rate through the silicon tubing for the best signal response in LabView™. Robustness studies on the probe should be completed, including autoclave tests and fermentation runs.

Ultimately, the methanol probe should be able to measure the methanol concentration in real time with an efficient response time and the sensor output can be used to control the methanol concentration during 30L fed batch *Pichia pastoris* fermentations to achieve high cell densities. First, scale up fermentation studies with *Pichia pastoris* will be needed. This can be accomplished using the 2L bioreactors and then scaled up to the 30L. A methanol feeding system should be implemented utilizing a peristaltic pump and load cell in order to feed the correct amount of methanol to the growing cells in the 30L bioreactor. Experiments will need to be designed and completed to determine the optimum feeding rate and how best to inject the methanol into the bioreactor. Furthermore, transfer studies of the culture from a seed 2L bioreactor to the 30L bioreactor will need to be completed. The 2L bioreactor in BTEC lab 218 is not a pressurized vessel and this will have to be considered while using a pump to feed the culture to the larger vessel. The gas exhaust monitoring system is available for use on the 30L bioreactor and could monitor inlet and exhaust gases while the system is in use.

After these goals are accomplished a transformed strain of *P. pastoris* will be used to express recombinant proteins. There are many phases of this project to continue in the future and I look forward to the success of future students.

## REFERENCES

- Arfman N., L. Dijkhuizen, G. Kirchhof, W. Ludwig, K. Schleifer, E.S. Bulygina, K.M. Chumakov, N.I. Govorukhina, Y.A. Trotsenko, D. White, and R.J. Sharp (1992). "*Bacillus methanolicus* sp. nov., a New Species of Thermotolerant, Methanol-Utilizing, Endospore-Forming Bacteria," *International Journal of Systematic and Evolutionary Microbiology*, 42, 439-445.
- Austin, G. D., S.K. Sankhe, and G.T. Tsao (1992). "Monitoring and control of methanol concentration during polysaccharide fermentation using an on-line methanol sensor," *Bioprocess and Biosystems Engineering*, 7 (6), 241-247.
- Brautaset, T., Ø.M Jakobsen, K.D Josefsen, M.C Flickinger and T.E Ellingsen (2006). "*Bacillus methanolicus*: a candidate for industrial production of amino acids from methanol at 50°C," *Applied Microbiol Biotechnology*, 1, 22-34.
- Cueva, J. P. (2012). "Design and Construction of a Sensing and Control System for Methanol Feeding in 30L High Cell Density *Pichia pastoris* Fermentations." Thesis Report. North Carolina State University. Raleigh, NC. USA.
- Gutmacher, F. (2009). "Insights into carbon flux in *Bacillus methanolicus*. Thesis Report. Beuth Hochschule für Technik". Berlin, Germany.
- Invitrogen Co. (2002). "Pichia Expression Kit: A manual for expression of recombinant proteins in *Pichia pastoris*," San Diego, CA, USA.
- Leak, D.J. (2010). "Methylotrophs, Industrial Applications," *Encyclopedia of Industrial Biotechnology, Bioprocess, Bioseparation, and Cell Technology*, 1-7, 3373-3383. John Wiley & Sons.
- Lee, G.H., W. Hur, C.E. Bremmon, and M.C Flickinger (1995). "Lysine Production from Methanol at 50°C Using *Bacillus methanolicus*: Modeling Volume Control, Lysine Concentration, and Productivity Using a Three-Phase Continuous Simulation," *Biotechnology and Bioengineering*, 49, 639-653.

Lindner, R. (2007). "Construction of an airlift reactor and comparison with STR for fed batch growth of *Bacillus methanolicus* on methanol," Thesis Report. Beuth Hochschule für Technik". Berlin, Germany.

Pingzuo, L., A. Anumanthan, X. Gao, V.V. Suzara, N. Duzgunes and V. Renugopalakrishnan (2007). "Expression of Recombinant Proteins in *Pichia Pastoris*," *Applied Biochemistry and Biotechnology*, 142, 105-124.

Pollard, D. and J. Christensen (2010) "Methylotrophs, Industrial Applications," *Encyclopedia of Industrial Biotechnology, Bioprocess, Bioseparation, and Cell Technology*, 1-7, 4759-4772. John Wiley & Sons.

Potvin, G., Ahmad, A., & Zhang, Z. (2012) "Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review," *Biochemical Engineering Journal*, 64, 91-105.

Schrader, J., M. Schilling, D. Holtman, M.V. Filho, A. Marx, and J. Vorholt (2009). "Methanol-based industrial biotechnoly: current status and future perspectives of methylotrophic bacteria," *Trends in Biotechnology*, 27(2), 107-115.

Tolner, B., L. Smith, R.H.J. Begent and K.A. Chester (2006). "Production of recombinant protein in *Pichia pastoris* by fermentation," *Nature Protocols*, 1(2), 1006-1021.

Vorholt, J. A. (2002). "Cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria," *Archives of Microbiology*, 178(4), 239-249.