ABSTRACT

CUEVA TELLO, JUAN PABLO. Design and Construction of a Sensing and Control System for Methanol Feeding in 30L High Cell Density *Pichia pastoris* Fermentations. (Under the direction of Dr. Michael Flickinger.)

Methylotrophs are organisms that grow on reduced carbon substrates such as methanol. Some methylotrophs like *Bacillus methanolicus* and *Pichia pastoris* have the ability to reach very high cell densities during fed-batch fermentation which may considerably improve product concentration on every production batch. However, high protein yields require an integral sensing and control system for the maintenance of optimal conditions inside a bioreactor's complex environment. The purpose of this study was to design and implement a reliable sensing and control system for methanol feeding by translating the knowledge from a 2L bioreactor model using *Bacillus methanolicus* into a 30L steam sterilizable bioreactor for *Pichia pastoris* cultivation. The design, construction and testing of an *in* situ silicone-tubing methanol probe was essential to complement the existing inlet and exhaust gassing system in the 30L bioreactor. The sensing and control system will utilize the manufactured probe and will be the basis for future research in high cell density cultivation of methylotrophic microorganisms. Throughout this project, skills including procurement of materials, cross functional communication and technical applications were gained.

Design and Construction of a Sensing and Control System for Methanol Feeding in 30L High Cell Density *Pichia pastoris* Fermentations

by
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DEDICATION

In loving memory of my father.

BIOGRAPHY

The author of this research was a fellowship award graduate student at North Carolina State University pursuing a Master of Science Degree in Biomanufacturing at the Golden Leaf Biomanufacturing Training and Education Center (BTEC). He is qualified by several years of experience in the design and optimization of biotechnological processes with special ability in data analysis and technical writing. Also, he has hands-on experience in development, scale-up and control of upstream and downstream production-scale bioprocesses in a cGMP environment for the biopharmaceutical industry. He obtained his BS from Escuela Politécnica del Ejército, Ecuador, where he headed a research project to determine the efficacy of silkworm extracts as hypoglycemic agents. Currently, he is a manufacturing scientist and works for Biogen Idec, a prestigious biologics large-scale manufacturing facility in RTP, North Carolina.

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I would like to thank Dr. Michael Flickinger for his valuable guidance throughout this research. I also would like to express my gratitude to Kit Yeung who helped me with several challenging technical tasks along the way. Special thanks to Driss Elhanafi, Michael Ray, Haiwei Zhang and Jennifer Sasser from BTEC staff who shared their knowledge and assisted with key aspects of this project. I am very thankful for the financial support from the National Science Foundation and BTEC during my studies in the Master's of Science in Biomanufacturing program.

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LIST OF ABBREVIATIONS

AOX	Alcohol oxidase
BSM	Basal salts medium
C_1	One-carbon compounds
CCP	Critical control parameters
CIP	Clean in place
COM	Serial communication port
DO	Dissolved oxygen
Daq	Data acquisition
HPLC	High-performance liquid chromatography
LabVIEW™	Laboratory Virtual Instrumentation Engineering Workbench
MeOH	Methanol
MGYH	Minimal glycerol medium + histidine
OD	Optical density
PCI	Peripheral component interconnect
SIP	Steam in place
VI	Virtual instrument
vvm	Vessel volumes per minute
WCW	Wet cell weight
XuMP	Xylulose monophosphate pathway
YNB	Yeast nitrogen base
YPD	Yeast extract, peptone and glucose medium.

1 INTRODUCTION

1.1 Heterologous gene expression in yeast

Methanol assimilation in Pichia pastoris

Methylotrophs are microorganisms that can grow on compounds without carbon-carbon bonds such as methane or methanol. Obligate methylotrophs specifically grow on one-carbon (C_1) compounds only. *Pichia pastoris* is a methylotrophic yeast, capable of using methanol as its only carbon source by using the xylulose monophosphate (XuMP) pathway. The diagram in Figure 1 shows the conversion of methane and methanol to C3 compounds by going different biosynthetic pathways:

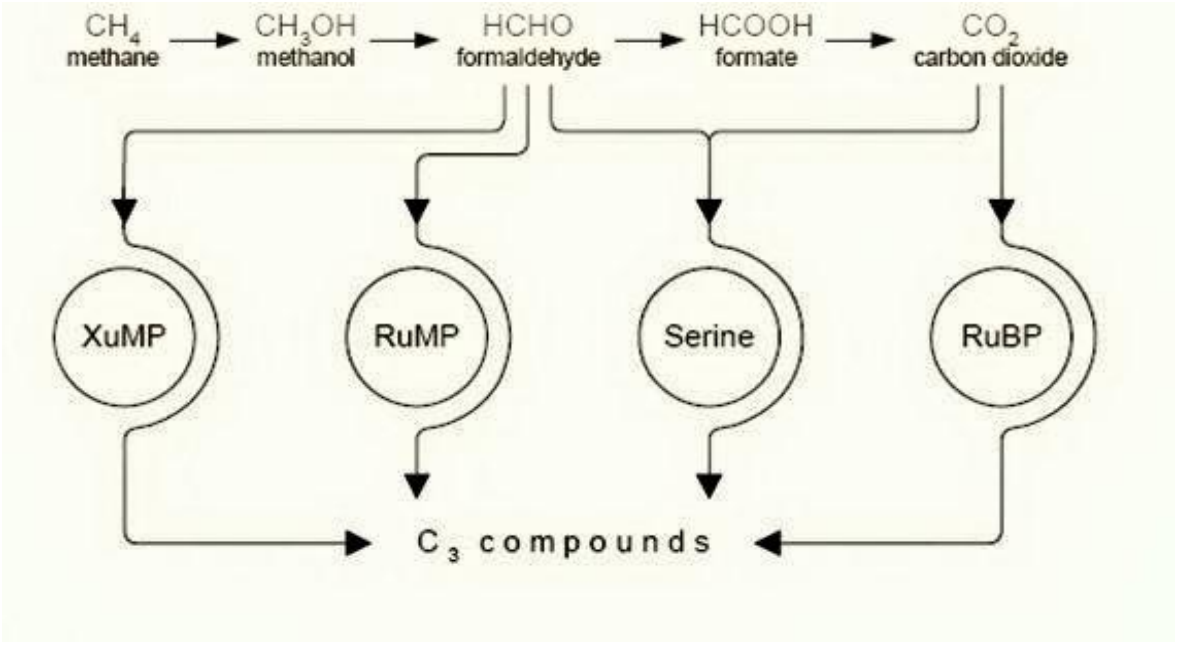


Figure 1. One-carbon assimilation pathways in methylotrophs
(Dijkhuizen *et. al.*,1992)

Hydrogen peroxide and formaldehyde are both generated from the oxidation of methanol through the catalysis of the enzyme alcohol oxidase (AOX). The methanol metabolism reactions take place in a specialized cellular organelle known as the peroxisome in order to avoid hydrogen peroxide toxicity for the cell. Given that alcohol oxidase has a low affinity for oxygen, microorganisms such as *Pichia pastoris* compensates for this deficiency by generating large amounts of the enzyme. Therefore, heterologous protein expression in *Pichia* can be regulated by the same cellular mechanism as the regulation of alcohol oxidase (Invitrogen Co., 2002).

Advantages of the Pichia pastoris expression system

Pichia pastoris is a well-suited microorganism for high cell density growth and has the ability as other eukaryotic systems of protein processing, protein folding and posttranslational modifications. In addition, it is faster and less expensive to grow than insect and mammalian cell cultures and is easy to genetically manipulate such as *Escherichia coli* and *Saccharomyces cerevisiae*. Moreover, the ability to reach very high cell concentrations can significantly improve overall protein yields (Pingzuo *et al.,* 2007; Invitrogen Co., 2000). It has been observed that *Pichia pastoris* can reach 10-to-100-fold higher heterologous protein concentration levels when compared to *Saccharomyces cerevisiae* making this host particularly desirable (Buckholz and Gleeson, 1991). Table 1 summarizes the advantages of using *Pichia pastoris* as the expression system for heterologous gene expression:

Table 1. Advantages of *Pichia pastoris* over other eukaryotic and prokaryotic expression systems. (Adapted from Pingzuo *et al.,* 2007)

	Advantage in P. pastoris expression systems	*Is a concern with…*
1	High cell density fermentation with relatively rapid growth rate.	Mammalian Cells
2	High productivity (g protein/L) in defined media suitable for biomanufacturing.	Mammalian Cells
3	No endotoxin or bacteriophage contamination.	Prokaryotic cells
4	Well-characterized yeast expression vectors.	Mammalian Cells
5	Viruses that infect on *P. pastoris* are free of pathogenicity in humans.	Mammalian Cells
6	Post translational modifications.	Prokaryotic cells
7	The ability to secrete proteins to the growth medium.	Prokaryotic cells

The AOX1 promoter

Expression in *Pichia pastoris* is controlled by a methanol-inducible promoter allowing high cell density biomass to be generated before induction. Linear DNA can yield stable transformants via homologous recombination with high stability even with multiple copies in the absence of selective pressure (Cregg *et al,* 1989).

The *AOX1* promoter has a well-known genetic sequence used widely in research and commercial applications since its patent usage rights have already expired (Sreekrishna, 2010) and is part of the expression cassette in *Pichia*. The *AOX1* and *AOX2* genes code for alcohol oxidase in *Pichia* and are regulated by methanol concentration. Therefore, the *AOX1* gene was isolated and the *AOX1* promoter is commonly used to drive expression of heterologous genes (Ellis *et al.,* 1985). A mature mRNA structure derived from the above mentioned expression cassette, ensures that the yeast cellular machinery recognizes the genetic sequence as familiar and does not contain cryptic sequences that may affect message stability or translational efficiency (Romanos *et al.*, 1992). Figure 2 shows the result of inserting a plasmid into linear DNA.

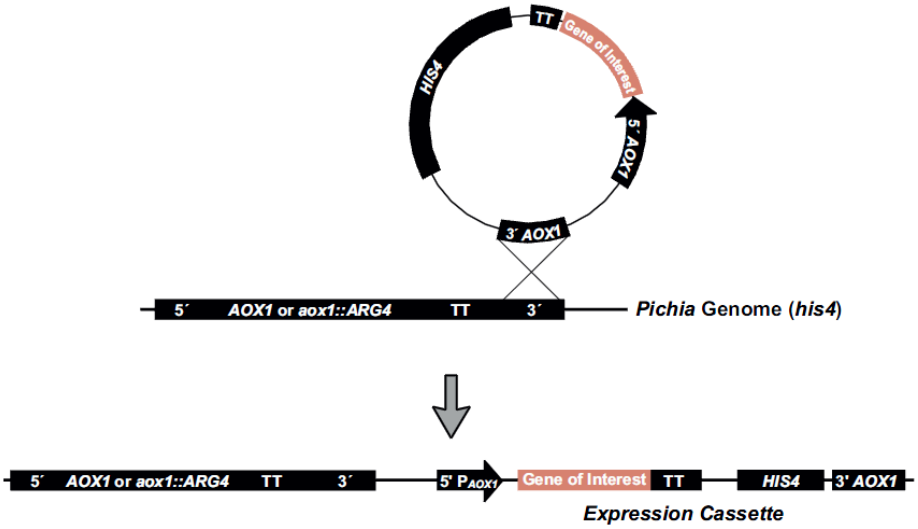


Figure 2. Insertion of the plasmid 3' to the intact *AOX1* locus (Mut$^+$).
(Invitrogen Co., 2002)

A disadvantage of using the *AOX1* promoter in a large manufacturing facility is the potential fire hazard when storing large quantities of methanol and all the associated safety requirements. Even though methanol toxicity is not a concern for the integrity of therapeutic products, there are alternative patent-protected *P. pastoris* promoters available that do not use methanol as the inducer and can be licensed from companies (Pingzuo *et al,* 2007; Sreekrishna, 2010).

Phenotype of AOX1 mutants

The ability to utilize methanol as carbon source in *Pichia pastoris* is related to its phenotype. There are three *AOX1* phenotype mutants: Mut$^+$ that utilizes methanol at a high rate, Mut^S which has the *AOX1* gene deleted and utilizes methanol at a low rate by using the *AOX2* promoter, and Mut$^-$ which is the minus phenotype with both the *AOX1* and *AOX2* genes deleted. Mut^S strains are desirable when methanol is considered a fire hazard and it requires less methanol during an industrial process

(Pingzuo *et al*, 2007). The most commonly used expression host of *P. pastoris* is GS115 (*his4*) which has a Mut^+ phenotype (Cregg *et al*., 1985). Table 2 shows the different properties for various phenotype strains of *Pichia pastoris*.

Table 2. Methanol requirements and specific growth rate of *P. pastoris* strains (Li *et al.,* 2007)

Strain type	AOX gene	Residual methanol (%)	Specific growth rate (h^{-1})	Methanol feeding rate
Mut^+	AOX_1^+, AOX_2^+	<0.5	0.14	+++
Mut^s	AOX_1^-, AOX_2^+	0.2–0.8	0.04	++
Mut^-	AOX_1^-, AOX_2^-	0.5	0.0	–

AOX_1^+, AOX_2^+: Strain has AOX_1 and AOX_2 genes

AOX_1^-, AOX_2^-: Strain does not have AOX_1 and AOX_2 genes

+++ = higher feeding rate, ++ = high feeding rate, – = no methanol feeding

Fed-batch fermentation with P. pastoris

Pichia pastoris can be grown in fed-batch mode by applying a two-phase fermentation process. During the first phase, the microorganism grows in batch mode with glycerol as the carbon source. This first phase is maintained until glycerol is depleted. The second phase includes the addition of a methanol feed so cells can be induced for heterologous protein expression. The level of recombinant protein expression in *Pichia* is comparable to that of *Escherichia coli* fermentation processes and considerably higher than *Saccharomyces cerevisiae* (Romanos *et al.,* 1992) with the ability to secrete post-translationally processed proteins into the medium. Factors such as medium composition and strain type can impact productivity. In addition, fermentation strategy and control parameters are important to increase productivity (Pingzuo *et al.*, 2007). It has been observed that the optimum temperature for *Pichia pastoris* fermentation is rather low in the range of 15°C to 30°C, which is an advantage to minimize extracellular proteolysis and promote

enhanced periods of protein production (Curvers *et al.*, 2001). Table 3 summarizes *Pichia pastoris* fermentation parameters:

Table 3. Summary of *Pichia pastoris* fermentation parameters
(Adapted from Invitrogen Co., 2002)

Parameter	Reason
Temperature (30.0°C)	Growth above 32°C is detrimental to protein expression
Dissolved oxygen (>20%)	*Pichia* needs oxygen to metabolize glycerol and methanol
pH (5.0-6.0 and 3.0)	Important when secreting protein into the medium and for optimal growth
Agitation (500 to 1500 rpm)	Maximizes oxygen concentration in the medium
Aeration (0.1 to 1.0 vvm for glass fermenters)	Maximizes oxygen concentration. Oxygen feed might be needed for high-cell densities
Antifoam (the minimum needed to eliminate foam)	Excess foam may cause denaturation of secreted protein and it also reduces headspace
Carbon source (variable rate)	Must be able to add different carbon sources at different rates during the course of fermentation

1.2 Bioreactor instrumentation and control

Monitoring and control of active fermentations

The degree at which the optimal conditions for microbial growth and product formation in a bioreactor can be maintained is a function of the control of critical process parameters (CCP) (Shuler and Kargi, 2001). The information generated by monitoring the process is of high value since it can be used by automated systems to correct CCPs and provide the best environment for cells to grow. Thus, the need of dynamically accurate (able to measure true values in changing conditions), reliable models and appropriate on-line sensors to provide a state of control in an active fermentation process is evident.

On-line and in-line measurements of process parameters can be performed with various techniques. For instance, insertable *in situ* steam sterilizable probes are the most common but use invasive methods that are a potential risk for bioreactor contamination. Non-invasive methods such as vent-gas analyzers and measurements from liquid slip streams are also an alternative. However some of these methods such as mass spectrometers can be costly and have not been used for complex molecules (Shuler and Kargi, 2001). Table 4 shows several approaches to monitor and control a bioreactor's chemical environment:

Table 4. Approaches to sensing and control of a fermenter's chemical environment (Adapted from Shuler and Kargi, 2001)

Approach	Possible measuring devices	Monitored Parameter	Comments
Insertable probes	pH electrode	H^+	Used extensively
	Galvanic and polarographic O_2 probes	pO_2	Measures partial pressure of oxygen, extended fermentations can cause drift and fouling.
	CO_2 probes	CO_2	Measures concentration of dissolved CO_2
	Fluorescence probes	NADH	Fouling
	Capacitance probes	Biomass	
	Biosensors	Wide range of compounds (e.g. ammonia, glucose, acetate, penicillin, etc)	Sterilization is challenging, not suitable for real time monitoring.
Vent-gas analyzers	Paramagnetic analyzer	O_2	Sample conditioning important
	Thermal conductivity or long-path infrared analyzer	CO_2	Sample conditioning important
	Flame ionization detector	Volatile organics (e.g. methanol)	Measures total hydrocarbon
	Mass spectrometer	O_2, CO_2, volatile substances	Highly specific but expensive.
	Semiconductor gas sensors	Organic vapors	Has been used primarily for ethanol
Measurements from liquid slip streams	HPLC	Dissolved organics. Proteins	Highly specific. Long response time. Expensive
	Mass Spectrometer	Dissolved compounds that can be volatilized	Highly specific but expensive.
	Enzymatic methods	Glucose	Slow response, limited acceptance

Methanol on-line sensing and control

Due to the strict regulatory environment that the biopharmaceutical industry is constantly challenged with, consistency from batch to batch is an important consideration. Therefore, high-level control strategies must be implemented in bioprocessing. Many of the control strategies make use of computers in closed-loop systems based on estimates of secondary parameters. For example, cell density can be estimated by measuring the off-gas composition and dissolved CO_2 concentration with a mass spectrophotometer. The computer estimates cell concentration with the above information and mass balance calculations. However, this strategy can be prone to error accumulation (Shuler and Kargi, 2001).

A common strategy for methanol-feeding rate control is the monitoring of the dissolved oxygen value combined with the knowledge of growth kinetics. The change of the dissolved oxygen concentration indicates when an increase or decrease in methanol feeding should be performed. Even though this strategy has been proven effective (Minning *et al.,* 2001), the dissolved methanol concentration cannot be precisely calculated via the dissolved oxygen and therefore, the dissolved methanol concentration may exceed the growth limiting values (Li *et al.,* 2007).

The advantage of on-line monitoring strategies such as an insertable dissolved methanol probe or an in-line gas analyzer for O_2 and CO_2 (BlueSens™™) is that systematic error can be mitigated and the response time improved. Both strategies require no sampling or pre-treatment of sample and allow for simple automated process control.

2 PROJECT GOALS

The purpose of this research was to design and implement a reliable sensing and control system for dissolved methanol concentration in high-density, fed-batch fermentations. The system will be further used for research in the production of recombinant proteins in a 30L stainless steel bioreactor. The above problem will be approached by translating the knowledge from an existent 2L glass bioreactor model using *Bacillus methanolicus* which had a completely functional sensing and control system for methanol.

2.1 General Objectives

- To understand and learn the existing 2L *Bacillus methanolicus* methanol feed control system and to be able to program a work schedule including ordering all required parts, installation and setup of the system.
- To design an on-line methanol probe compatible with the stainless steel 30L reactor geometry and its environment.
- To construct a stainless steel probe based on the above design that allows sensing a voltage signal to correlate and control the bioreactor's dissolved methanol concentration.

  To design and implement the methanol sensing and control system in a 30L stainless steel bioreactor after procuring all necessary parts.
- To install and learn the programming and utilization of the LabVIEW™ (Laboratory Virtual Instrumentation Engineering Workbench) platform software to visualize the analog signal from the sensor probe.
- To assess the methanol probe response and describe a procedure to calibrate the instrument.
- To grow a high-density culture of *Pichia pastoris* in order to calculate the methanol flow rate range for this organism when using a specific strain.

3 EXPERIMENTAL METHODS

3.1 Microbial cultures

Media preparation

Chemically defined media and YPD were used to culture *Pichia pastoris* BEC7. A minimal glycerol medium + histidine (MGYH) was also used for growing starter cultures. YPD medium is a complex medium often used for growing *S. cerevisiae*. It is composed of yeast extract (for vitamins and other nutrients), peptone (peptides derived from enzymatically digested proteins), and glucose (the energy source for the yeast). However, this medium is not suitable for drug production due to its animal components and was used as a reference only. In contrast, chemically defined media is appropriate for therapeutic product manufacturing and was also tested for *Pichia pastoris* growth.

YPD media can be stored at room temperature with a shelf life of several months. The recipe for YPD media used in this study is shown in Table 5.

Table 5. YPD medium for yeast.
(Invitrogen Co., 2002)

Yeast extract	10g
Bacto-peptone	20g
Dextrose (glucose)	20g
Tryptophan	0.3g
Distilled water	1000mL

For MGYH media preparation, 800mL of autoclaved water was combined with 100mL of 13.4% yeast nitrogen base (YNB) with ammonium sulfate without amino acids, 2mL of 0.02% biotin solution, 100mL of 10% glycerol solution, and 10mL of

0.4% histidine. The media was stored at 4^{o}C until use. The final composition of MGYH medium is shown in Table 6.

Table 6. Minimal glycerol + histidine medium composition (Invitrogen Co., 2002)

YNB	1.34%
Glycerol	1%
Biotin	4 x 10^{-5}%
Histidine	0.004%

For the chemically defined medium preparation, the appropriate amount of fermentation basal salts and PTM_1 Trace salts solutions were prepared. 75mL per liter initial fermentation volume of 50% glycerol containing 12mL PTM1 trace salts was added to the fermentation basal salts medium and then filter sterilized. The recipes are shown in Table 7 and Table 8.

Table 7. Fermentation basal salts medium recipe (Invitrogen Co., 2002)

Phosphoric acid, 85%	26.7 mL
Calcium sulfate	0.93 g
Potassium sulfate	18.2 g
Magnesium sulfate-$7H_2O$	14.9 g
Potassium hydroxide	4.13 g
Glycerol	40.0 g
Water	to 1 liter

Table 8. PTM_1 trace salts solution
(Invitrogen Co., 2002)

Cupric sulfate-$5H_2O$	6.0 g
Sodium iodide	0.08 g
Manganese sulfate-H_2O	3.0 g
Sodium molybdate-$2H_2O$	0.2 g
Boric Acid	0.02 g
Cobalt chloride	0.5 g
Zinc chloride	20.0 g
Ferrous sulfate-$7H_2O$	65.0 g
Biotin	Biotin
Sulfuric Acid	5.0mL
Water	To 1 liter

Cryoconservation of Pichia pastoris

Pichia pastoris Mut^+ was plated on YP base agar medium to form colonies. After 24 hours of growth, an isolated colony was scraped and used to inoculate 50mL of YPD media in a 250mL baffled shake flask. The culture was incubated at 30°C and 300rpm for 12 hours until the OD_{600} was in the range of 1.2 to 1.8. Then, 32mL of the above culture was mixed with 8mL of 50% glycerol and aliquoted into 1mL sterile vials. The vials were stored at -70°C until use.

Cultivation of Pichia pastoris in shaker flasks and growth curves

Pichia pastoris Mut^+ BEC6.1 and BEC7 strains were cultivated in baffled 250mL Erlenmeyer shake flasks with milk filter disk closures into a final volume of 50mL using chemically defined and YPD media. First, starter culture is grown from cryopreserved stocks in minimal glycerol + histidine medium in order to acclimatize cells from the frozen shock. Once they reached the exponential phase after an

overnight period or 16 to 24h, an appropriate amount of cells based on OD_{600} readings were transferred into 50mL of either chemically defined or YPD media to a final concentration of 0.1 OD_{600}. Growth conditions were 30°C and a rotational speed of 300rpm. In order to maintain the viability of cultures and prevent contamination, all work was conducted using aseptic conditions in a vertical laminar flow biological safety hood. OD_{600} measurements were performed every 2 hours in order to assess for cell concentration. To accurately measure cell density, samples with OD_{600} higher than 1.0 were diluted down before reading.

3.2 Methanol sensing and control system

Description of the 2L bioreactor system

A 2L scale-down model was used for the design and implementation of a methanol feed sensing and control system into a 30L bioreactor setting. The small-scale model is a self-constructed autoclavable 2L bioreactor, P140 series (Applikon, Schiedam, Netherlands) that utilizes a diverse set of probes, sensors and mass flow controllers connected to a single data acquisition system for fermentation monitoring and control. The geometry of the bioreactor was measured and is represented in Figure 3.

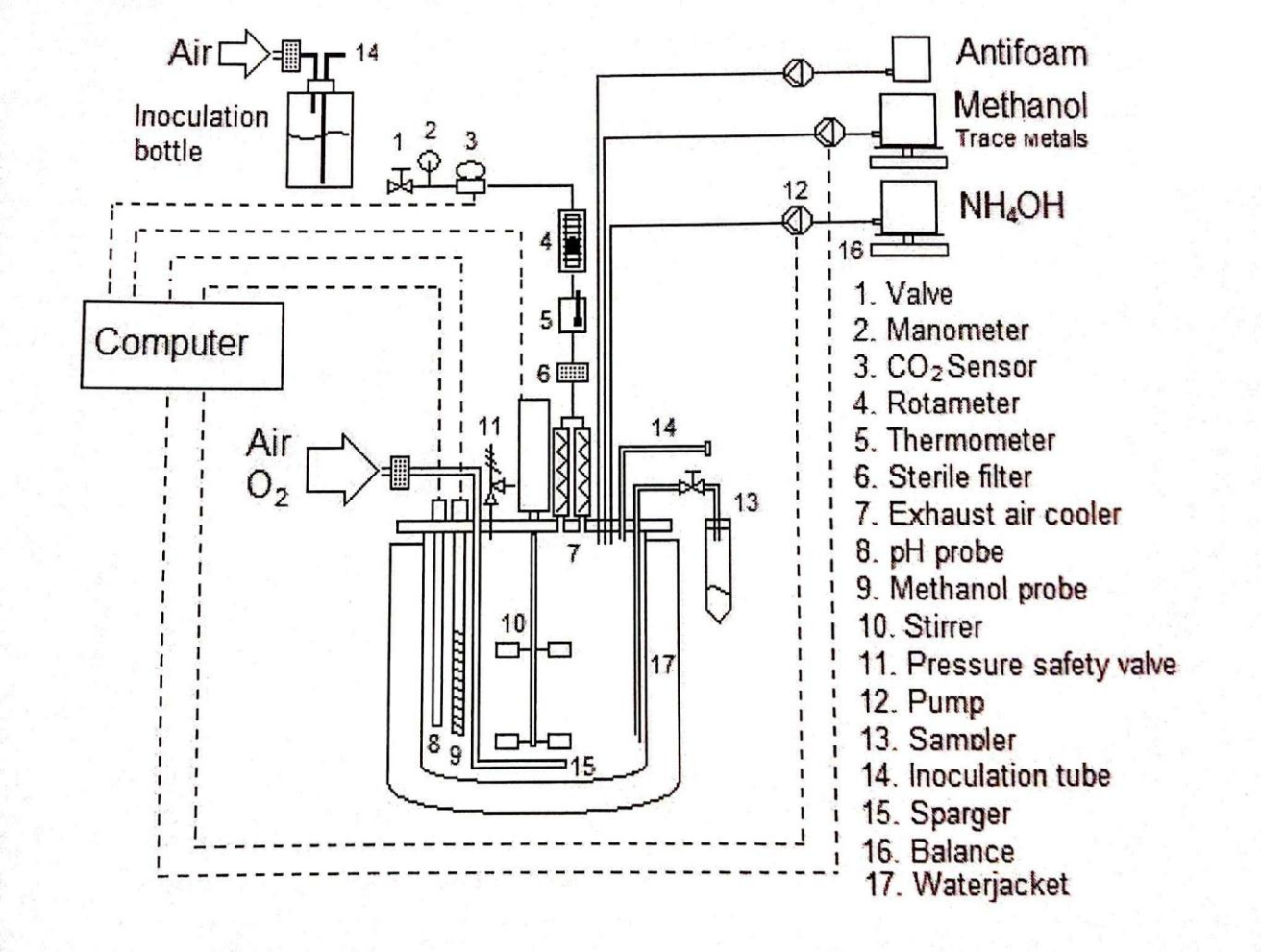


Figure 3. Small-scale 2L bioreactor schematic

(Gutmacher, 2009)

A data acquisition system (LabVIEW™ 8.6, National Instruments) receives analog and digital signals for process monitoring. The software is also a platform for instrument control and automation. Table 9 summarizes all types of signals managed by the small-scale model.

Table 9. Two-liter system analog and digital signals acquired with LabVIEW™ (Hart, 2010)

Analog Signals	Digital Signals	Signals read manually
Temperature	Exhaust gas CO_2 concentration	Thermometer (exhaust gas)
pH	Inlet air flow rate	Scale (base mass)
Inlet oxygen flow rate		Gauge (back pressure)
Dissolved O_2 concentration		Gauge (outlet pressure of the nitrogen tank regulator)
Agitation speed		
Exhaust gas flow rate		
Methanol concentration		

Digital signals are directly acquired by serial communication (COM) in the back panel of a Dell Precision T3400 workstation. Analog signals are acquired with a peripheral component interconnect card (PCI-6229) with capacity for 32 analog inputs. The PCI card is connected to a shielded I/O connector block with 68 pin-connectors that accommodates all analog signal wiring into a single array of inputs.

The bioreactor temperature is controlled by an independent operating temperature controller. The circulating water within the bioreactor's jacket exchanges heat through the use of a heated bath and a cooling circulation system. The cooling system utilizes a pump controlled by the temperature controller to cool down the heat transfer fluid tempering box with non-freezing liquid of the cooling container. In the same manner, a solenoid valve is actuated to let hot water into the jacket. A thermocouple uses the bioreactor's thermowell on the head plate filled with water for data acquisition with LabVIEW™ and control with the independent temperature controller. Figure 4 shows a schematic representation of the temperature regulating system:

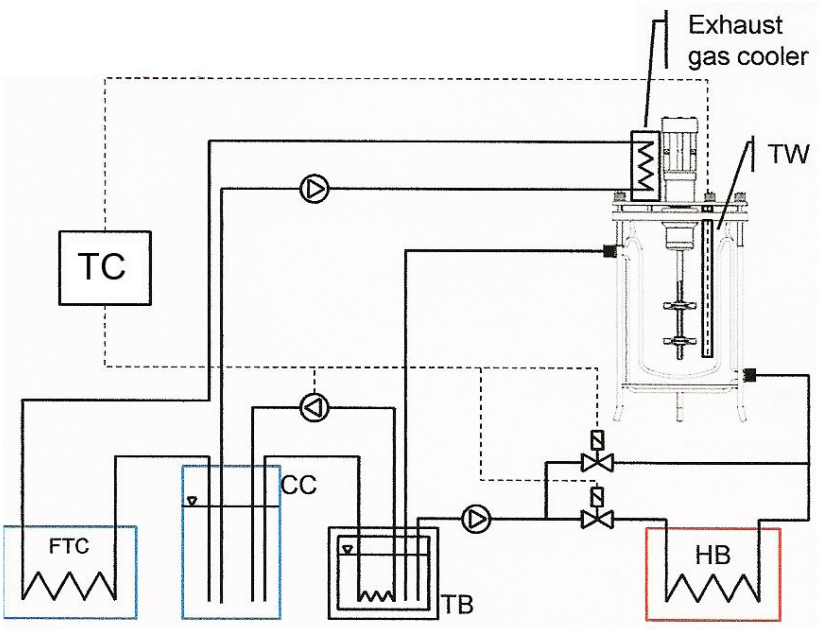
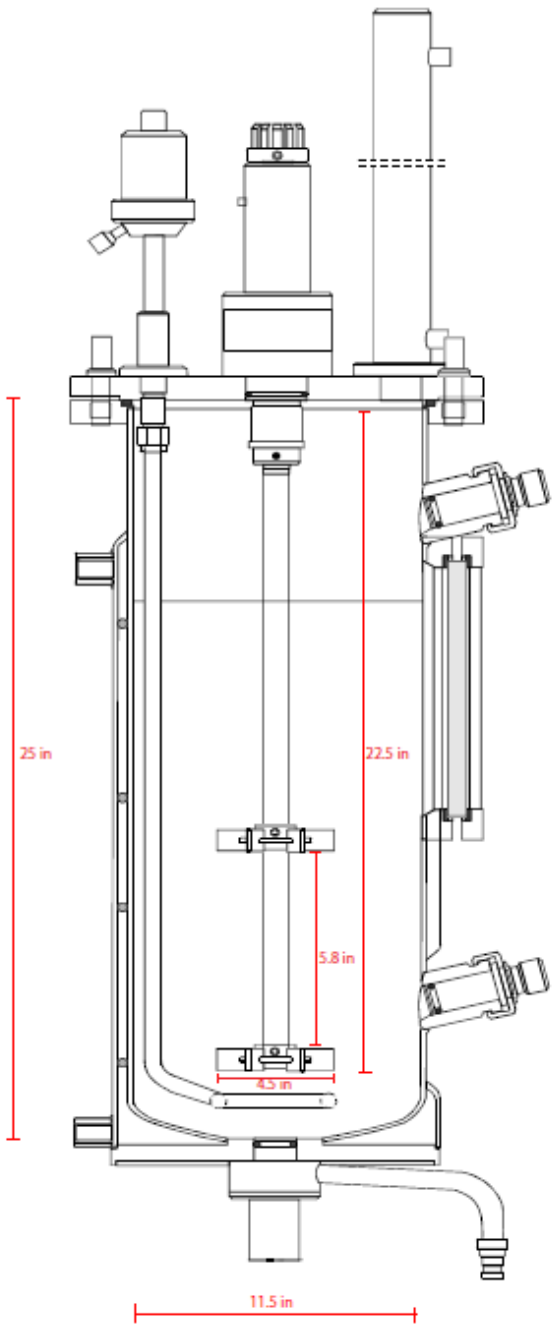


Figure 4. Temperature control scheme of the 2L bioreactor system
Solid lines = liquid flow; dashed lines = electrical control of solenoid valves; TC = Temp. controller; FTC = Flow through cooler; CC = Cooling container; B = Tempering box; HB = heat bath; TW = Thermowell with thermocouple. (Hart, 2010)

Proposed 30L bioreactor sensing and control system

A pilot-scale, 30L bioreactor system (Biostat® DCU3, Braun Biotech International) was modified for dissolved methanol concentration monitoring. A schematic diagram of the 30L bioreactor and its geometry measurements are shown in Figure 5. An existing inlet and exhaust gas lines panel using BlueSens™ technology (Herten, Germany) was already in place before this study (Figure 6). However the procurement of additional parts such as rotameters and mass flow controllers was expedited in order to complete the proposed sensing and control system shown in Figure 7.



Height/diameter ratio	2.2:1
Total volume	42 L
Working volume	30 L
Vessel internal diameter	29.2 cm
Vessel height	63.5 cm
Impellers	2
Distance between impellers	14.7 cm
Impeller diameter	11.4 cm
Baffles	4

Figure 5. Pilot-scale 30L bioreactor geometry.

(Actual measurements, bioreactor drawing adapted from B. Braun Biotech International, Sartorius Group)

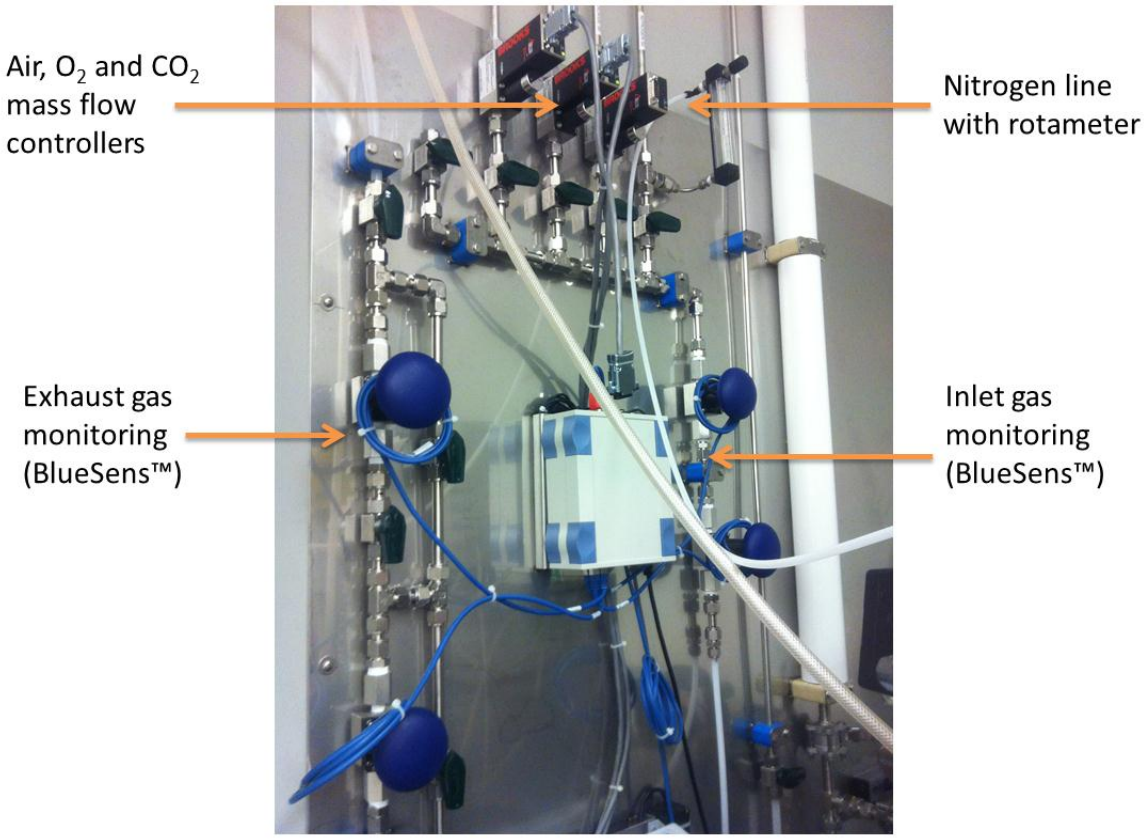


Figure 6. BlueSens™ sensors for inlet and exhaust gas monitoring

The proposed 30L bioreactor sensing and control system is mostly controlled by the computer attached to the 30L bioreactor which uses proprietary software to control process parameters. However, the dissolved methanol concentration sensing and feeding control was designed to be an independent closed-loop system. A nitrogen gas line regulated with a rotameter was installed for feeding inert nitrogen gas through the methanol probe. In addition, a connector box was used to house the circuit of a TGS-822 sensor for the detection of methanol vapors. The methanol sensor was placed near the probe to minimize the path length from where the methanol diffuses through the silicone tubing to the sensor to improve response time. The LabVIEW™ platform would function from an adjacent computer to sense and control methanol feed through the actuation of a peristaltic pump.

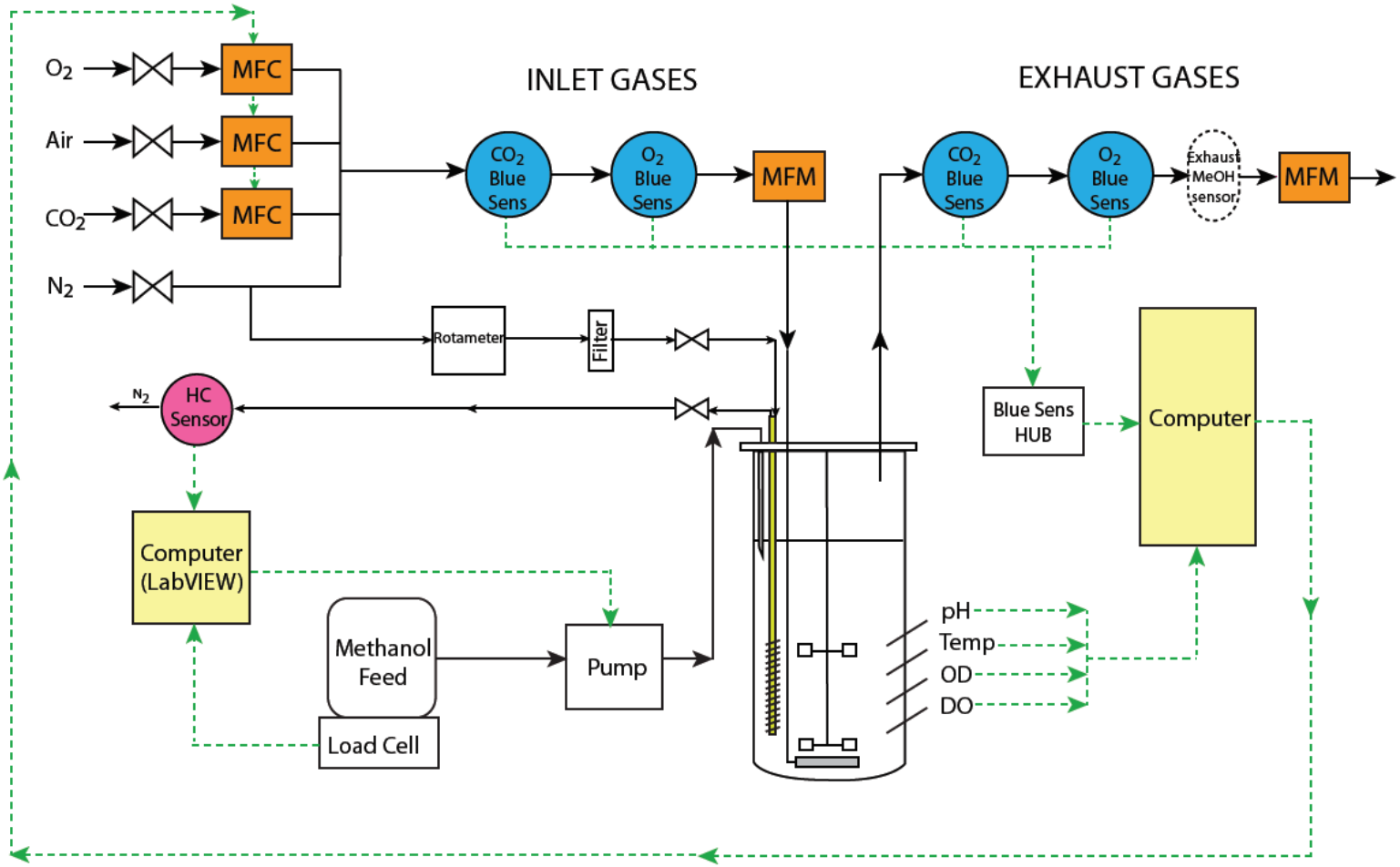


Figure 7. Proposed 30L bioreactor sensing and control system

Methanol probe design

In biomanufacturing, bioreactor sterile conditions are required in order to ensure the cultivation of an axenic culture. Clean in place (CIP) and steam in place (SIP) procedures are widely used in a biomanufacturing setting to prevent contaminating microorganisms that would consume nutrients and negatively impact the producer microorganism's growth. The utilization of on-line probes significantly increases the probability of contamination (Shuler and Kargi, 2001). Therefore, the benefit of adding a new probe to the fermentation system should outweigh the risk associated with contamination and the economic losses they might generate. The methanol probe design took into consideration the above described risk. The probe was designed to withstand high temperatures (121°C) and humidity levels of 100% with the use of 316 grade stainless steel. All seals and parts were assembled following a minimalist design to allow for cleanability and to minimize areas of contaminant accumulation.

The 30L bioreactor geometry was also taken into consideration for the design. A head plate probe was preferred since all four ports in the bioreactor probe penetration belt will be occupied with pH, DO and OD probes, including a free port for bioreactor sampling. However, sampling could also be performed using a dip tube from the head plate. The probe was constructed with a hollow stainless steel rod with two 1.59 mm o.d. diameter stainless steel tubes inside. One of the stainless steel tubes exits the probe through the side while the other tube exits at the bottom of the probe. 220 cm of laboratory-use silicone tubing (1.57 mm internal diameter x 2.41 mm outer diameter, wall thickness = 0.84mm) was spiraled around the stainless steel rod and each end was connected to one of the 1.59 mm tubes in such a way that the nitrogen gas will flow in one single path throughout the probe. The probe length and the silicone tubing placed at the bottom end of the stainless steel rod

allow operating at the minimum vessel volume (9L) and position the tubing into an adequate turbulent location. Thick-wall Tygon™ tubing was used to connect the probe gas outlet to the sensor box. Figure 8 provides a schematic of the dissolved methanol probe and shows the prototype fabricated by the machine shop.

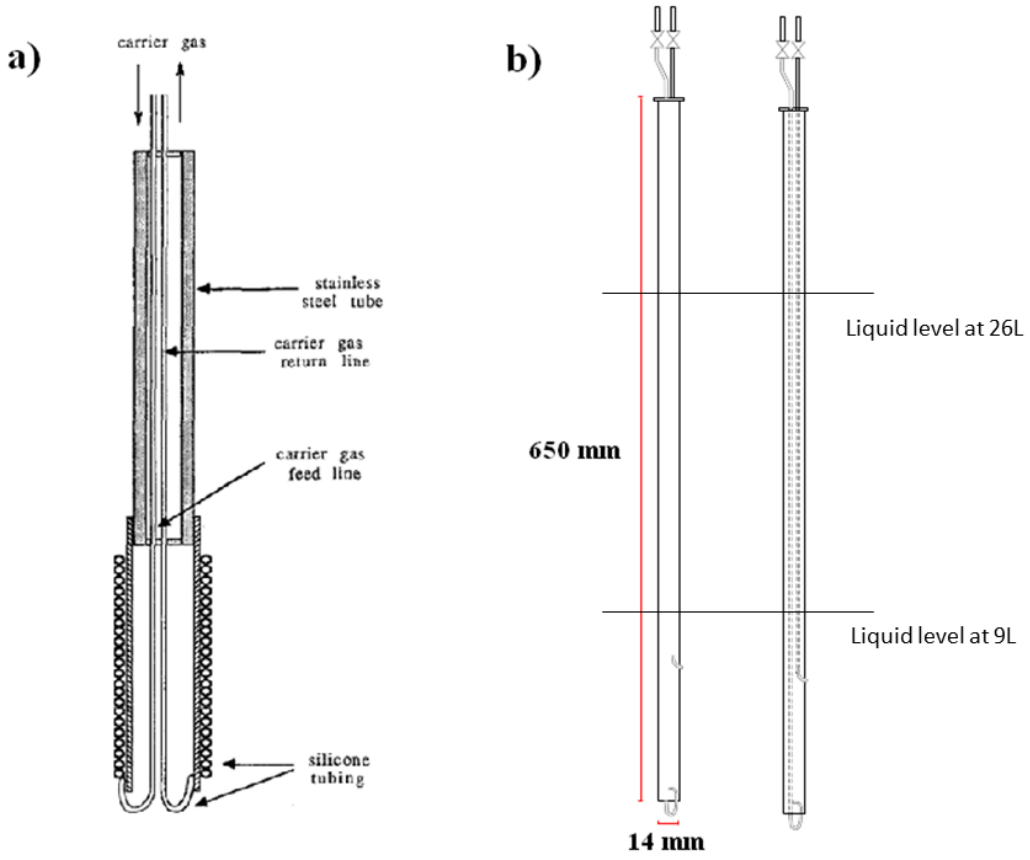


Figure 8. Dissolved methanol probe scheme and prototype
a) Methanol probe scheme (Adapted from Austin *et al*, 1992)
b) Head plate methanol probe prototype for 30 liter reactor

When comparing the 2L lab-scale model probe design with the 30L bioreactor methanol probe, most significant differences are the silicone tubing wall thickness and the exposed surface area. The 2L Lab-scale methanol probe has a tubing wall

thickness of 0.55 mm whereas the 30L bioreactor methanol probe tubing wall thickness is 0.84 mm. In the same manner, the exposed surface areas are 35.5cm^2 and 83.3cm^2 for the 2L and 30L dissolved methanol probe respectively. The tubing surface area per liter for each reactor is 35.5 cm^2/L (at 1L working volume) and 3.2 cm^2/L (at 26L working volume).

3.3 Sensor and data acquisition system setup

Methanol sensor circuit construction

A plastic connector box was used to house the circuit for a TGS-822 hydrocarbon sensor (Figaro USA, Wilmette, Illinois) for the detection of methanol vapors. This circuit box was built as a replica of the one installed in the 2L bioreactor system model. Figure 9 shows the circuit connections for the hydrocarbon sensor:

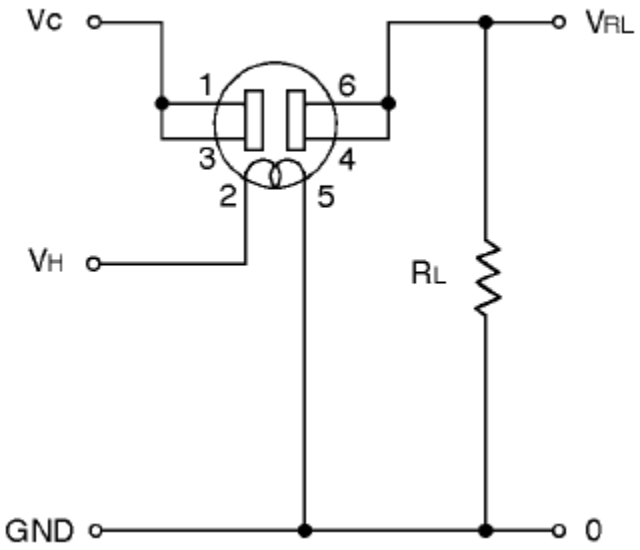


Figure 9. Hydrocarbon sensor basic circuit diagram

V_C = Circuit voltage Max. 24V AC or DC; V_H = Heater voltage 5.0 ± 0.2, DC only;

RL = Load resistance 0.45kΩ min. (Figaro USA).

The above circuit was powered with a multiple output voltage linear power supply (Power-One, model HTTA-16W-AG) according to the basic circuit diagram. The sensor was preheated for more than seven days before testing. This preheat time is necessary when the sensor's heating element has not been continuously connected to the 5V current from the power supply.

Data acquisition system

The independent closed-loop system for methanol sensing and control required of a data acquisition (Daq) system for analog signal acquisition. A peripheral component interconnect card (PCI-6229) with capacity for 32 analog inputs was installed with its respective drivers into an adjacent computer in the same room as the 30L bioreactor. The PCI card was connected to a shielded I/O connector block with 68 pin-connectors that accommodates all analog signal wiring into a single array of inputs. The connector block accommodated the 'V_{RL}' signal and ground reference from the sensor circuit box. The connection scheme is represented in Figure 10.

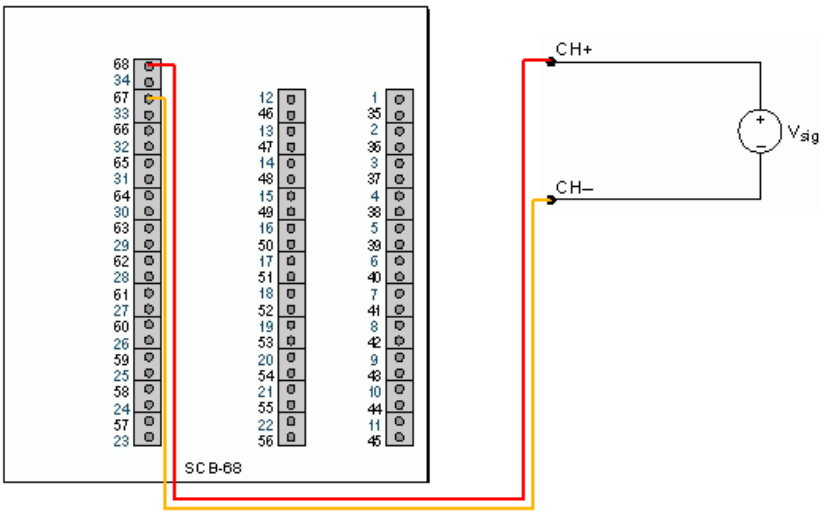


Figure 10. Shielded I/O connector block connections

Pins 68 and 67 corresponded to pins ‘AI 0’ and ‘AI GND’ respectively in the PCI card terminal (Connector 0) in ‘RSE’ mode (measurement made with respect to ground). This nomenclature is used within the configuration of the analog signal when using the ‘Measurements and Automation Explorer’ tool installed with LabVIEW™. All connection channels might be changed if necessary to include new signals. The PCI card terminals are represented in Figure 11.

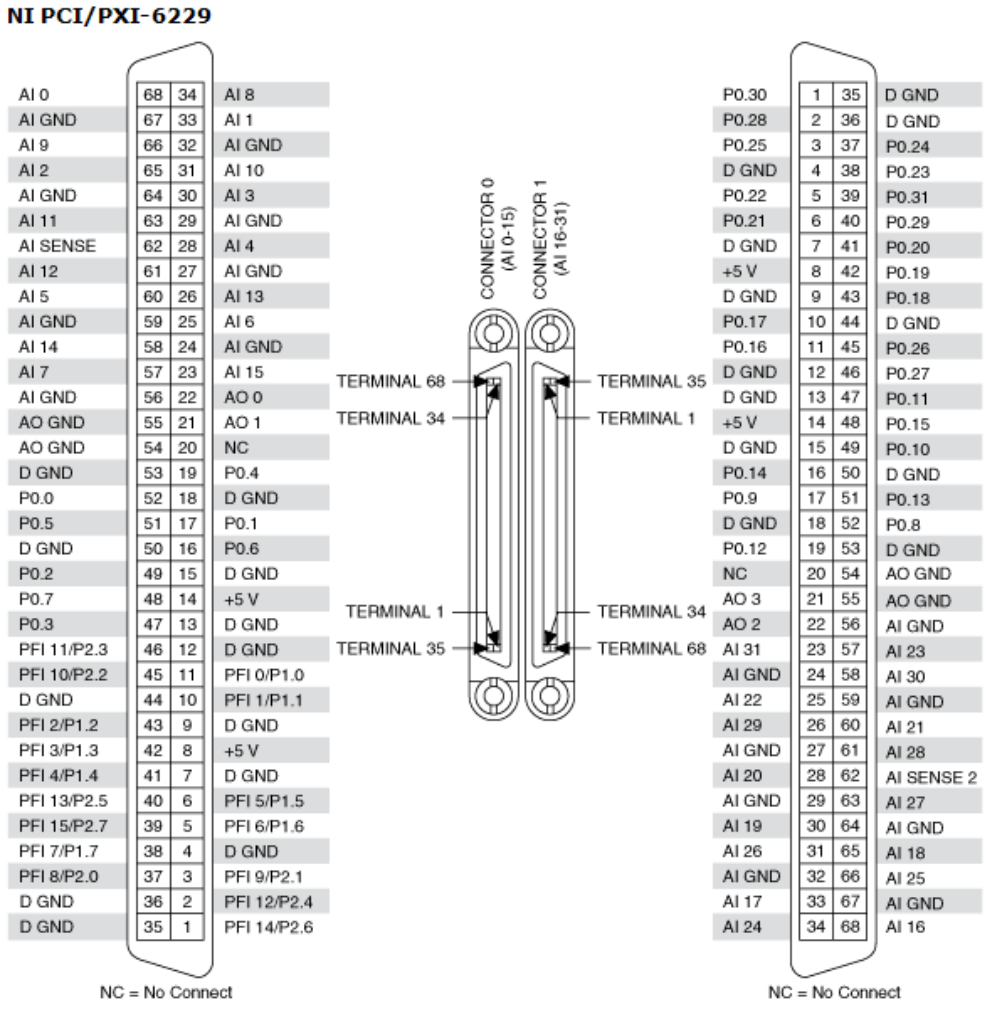


Figure 11. PCI -6229 card connector terminals map.

LabVIEW™ platform for documentation and control of methanol

LabVIEW™ 2011 programmable platform was utilized to write a program that allows the acquisition of a voltage signal from the TGS-922 sensor basic circuit and translate it to a graphical representation. LabVIEW™ automatically recognizes the installed PCI card drivers and the respective connector block.

4 RESULTS

4.1 Pichia pastoris growth rate

Pichia pastoris strain BEC6.1 was grown initially in chemically defined media in shake flasks. However, after successfully subculturing the strain in MGYH medium, the cells did not grow in minimal basal salts medium. Therefore, a different strain (BEC7) was evaluated for growth curves. Unfortunately, *Pichia pastoris* BEC7 did not grow in the chemical defined media as well, even though the medium was prepared in two separate types, one containing histidine and a second one with no histidine. Even though basal salts medium is the most common medium for high cell density fermentation for *Pichia pastoris*, it may not be the optimum due to its composition which results in precipitates when changing ionic strength, among other problems (Sreekrishna *et al.*, 2010). Since the purpose of this growth curve assay was to acquire information on the maximum specific growth rate of *Pichia pastoris*, YPD media was used instead to ensure proper growth. This time, the growth was successful and is shown in Figure 12.

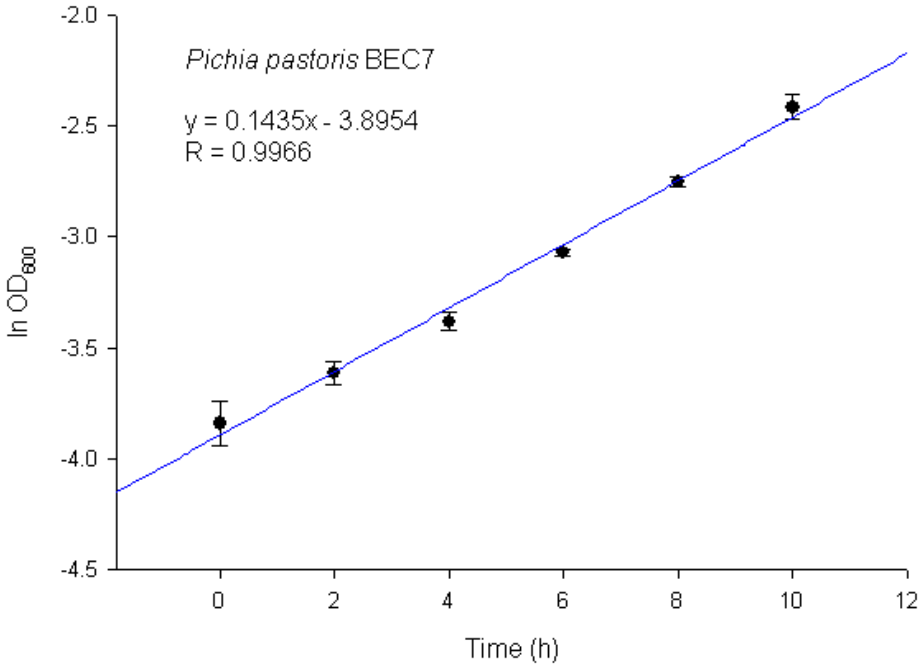


Figure 12. *Pichia pastoris* strain BEC7 growth curve on YPD medium

The semi-logarithmic plot shows a good fit (R = 0.9966) and a maximum growth rate of 0.146 h^{-1} on YPD medium.If the growth kinetics of a *Pichia* production strain are pre-established, then the maximum methanol feed rate can be adjusted according to the specific growth rate during the induction phase. This can be an effective way to maintain optimal methanol concentration in the bioreactor (Sreekrishna *et al.*, 2010). However, the culture may not always grow at this maximum growth rate on methanol. The maximum specific growth rate constant can be used to calculate the methanol uptake rate with Equation 1.

$$\mathrm{MeOHUR} = \frac{\mu_{max} \cdot \mathrm{X}}{\mathrm{Y_{X/MeOH}}}$$

Equation 1. Maximum methanol uptake rate calculation

$Y_{X/MeOH}$ = Biomass yield on MeOH; X = Biomass concentration

Pichia pastoris has been observed to reach cell densities of 450 g WCW/L in BSM medium supplemented with PTM1 trace salts in a quasi-steady state (Zhang *et al.,* 2011). Therefore, assuming cell concentrations in the range of 30g/L (Sreekrishna *et al.*, 2010) to 450 WCWg/L and a biomass yield on methanol of 1.19 g WCW/g MeOH (Zhang *et al.,* 2011), the methanol uptake rate value could be as high as 3.5 g MeOH/L·h to 52.9 g MeOH/L·h. Therefore, a dissolved methanol probe would need to sense changes in dissolved methanol concentration fast enough to respond within this timeframe to be useful to control dissolved methanol concentration.

4.2 Data acquisition system

A data acquisition system was installed and assembled in an adjacent PC computer for closed-loop control of methanol feed. A basic circuit was replicated from the 2L bioreactor system and is shown in Figure 13. The sensor was pre-heated with 5V DC for at least seven days before calibration. A stream of nitrogen gas was diverted from the main nitrogen line on the back panel and flowed through the probe and the basic circuit with the TGS-822 sensor during pre-heating time.

The voltage signal emitted by the circuit box was then wired into the National Instruments connector block along with a ground reference cable in order to record the data in a PC.

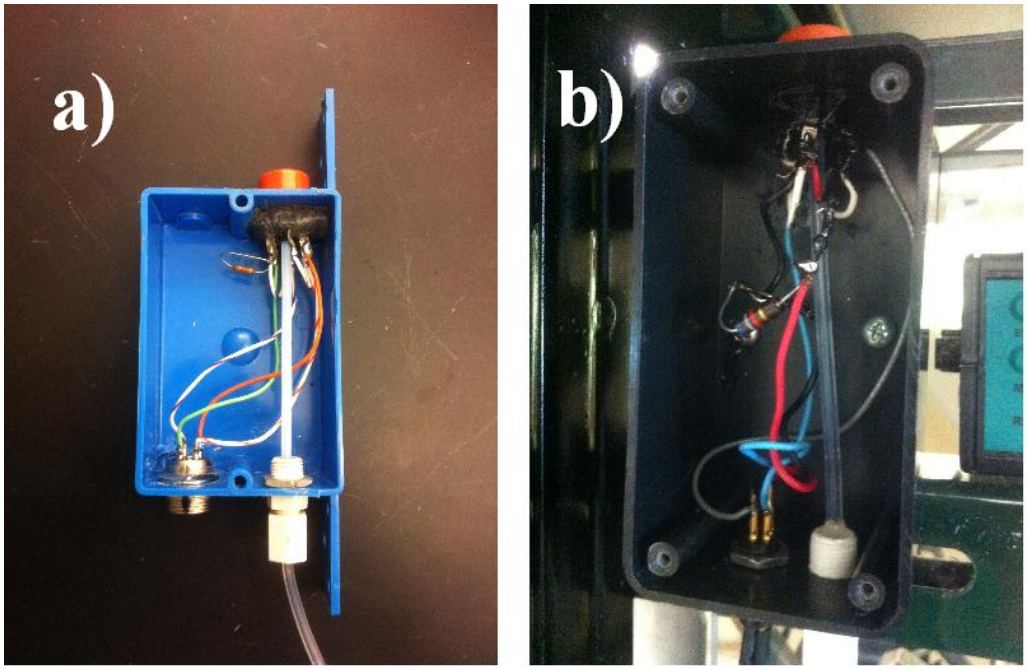


Figure 13. TGS-822 sensor basic circuit connection box
a) Circuit replica adapted for the 30L bioreactor system;
b) Original connection box from the 2L bioreactor model

A basic LabVIEW™ VI program was written using the graphical interface and is shown in Figure 14. The algorithm converts the analog signal in digital format and filters it before displaying it into a waveform chart at a sampling rate of 1000 hz. The program is able to store one sample per second but this parameter can be changed using the 'Daq Assistant' properties option.

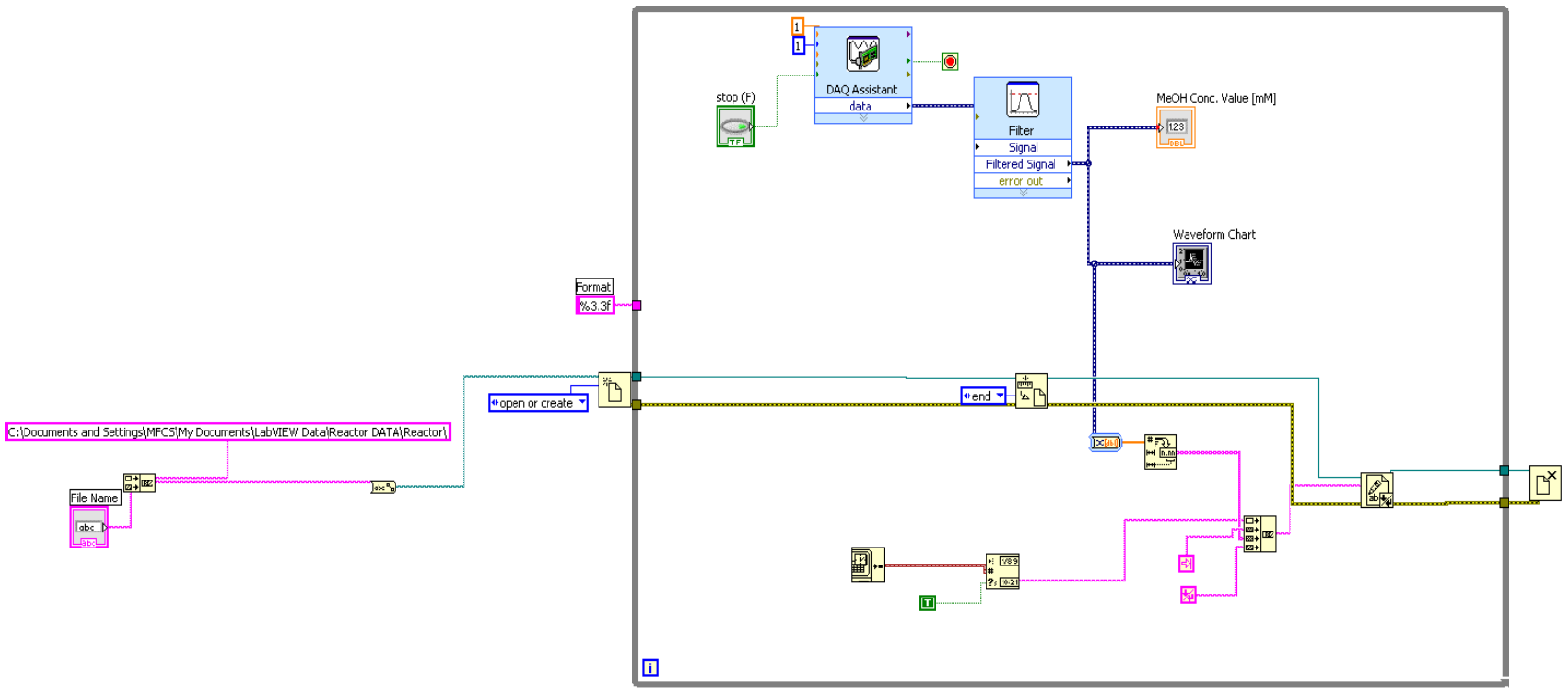


Figure 14. LabVIEW™ program for methanol data acquisition

Figure 15 shows the screen front panel interface of the programmed VI. It displays a numeric value for methanol concentration in mM and displays the data in a waveform chart that updates every second.

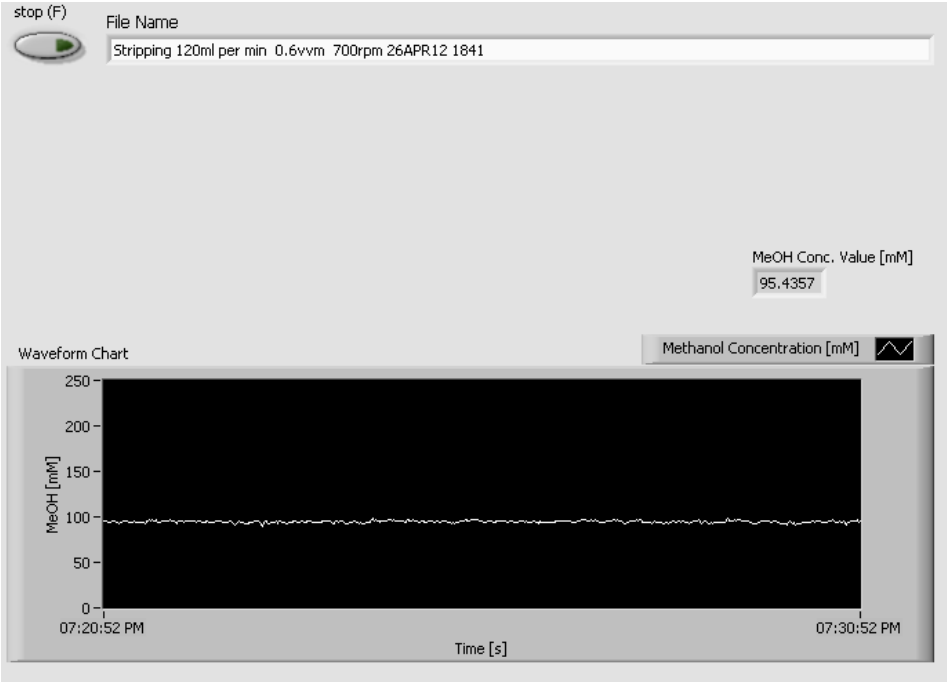


Figure 15. LabVIEW™ screen program front panel for methanol data acquisition

4.3 Methanol probe

The constructed methanol probe is shown in Figure 16. 220cm of silicone tubing were used which resulted in a calculated area of 83.3 cm^2. The tubing connections were secured with RTV sealant on both sides. It was possible to use a cable tie only in the bottom end connection otherwise any additional parts on the top connection end would increase the probe diameter. The probe was screwed onto the bioreactor's head plate as shown on Figure 17. The distance from the silicone coil to the sensor was 125 cm.

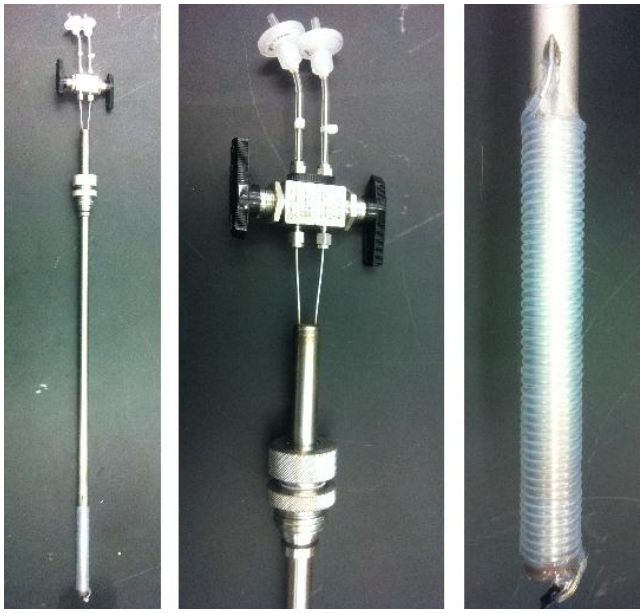


Figure 16. Dissolved methanol probe for the 30L bioreactor system

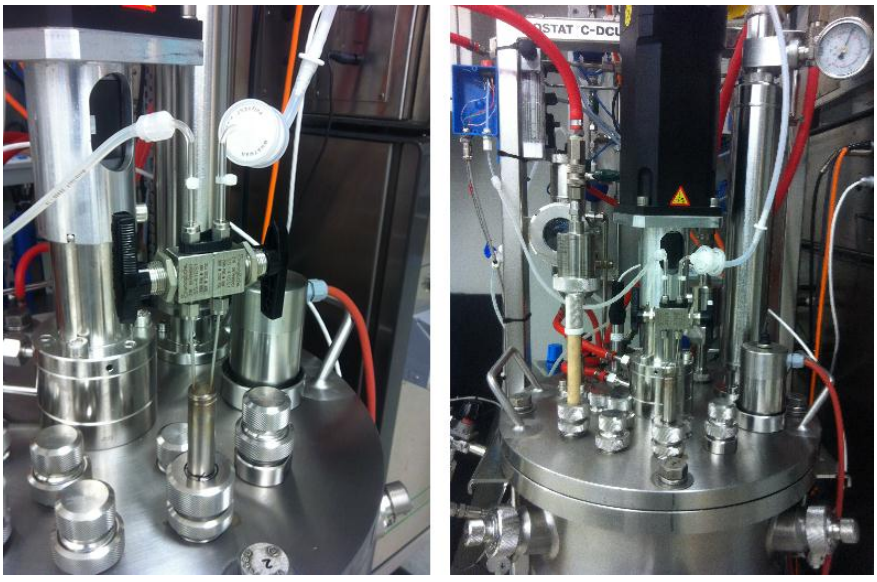


Figure 17. Methanol probe installed in the head plate of the 30L bioreactor

4.4 Methanol probe calibration

The analog signal from the sensor circuit was measured in volts. In order to translate the above voltages into a concentration value (mM), a calibration assay was performed. An appropriate amount of neat methanol was weighted and mixed with 50g of water. Then, it was poured to the bioreactor containing 26kg of water through a funnel using an opening port on the head plate. The final methanol concentrations were 0.00, 12.3, 24.6, 36.9, 49.0, 97.5, 146 194 and 241 mM that correspond to percentage values in the range of 0.05% to 1.00%. The aeration rate, the agitation rate and the temperature were set to 0.6 vvm, 275 rpm and 23°C respectively.

A built-in calibration wizard program was run to calibrate the probe (right-click on Daq assistant>Properties>Calibration>New Calibration) as shown in Figure 18. Before committing to a new calibration value each time additonal methanol was added, the voltage reading had to stabilize within ±5%. The calibration wizard program automatically scales the readings to mM after the calibration process is finished.

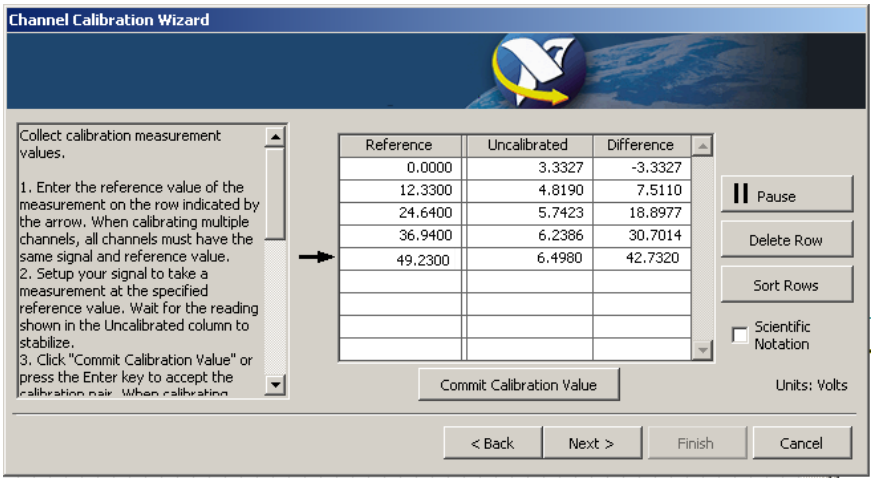


Figure 18. LabVIEW™ calibration wizard interface

The probe was calibrated using 120mL N_2/ min and 500mL N_2/min nitrogen flow at 275rpm and no air flow through the sparger. However, the ideal conditions should include air sparging to mimic normal operations. It was observed that the change in nitrogen flow rate affected the initial voltage signal, the slope of the calibration curve, and thus, the sensor's working voltage range. The sensing element is a tin dioxide semiconductor with low conductivity in clean air; any amount of organic gas would change this conductivity value. Therefore, it is suspected that the current house nitrogen gas line may not provide hydrocarbon-free "clean" nitrogen. Figure 19 shows the calibration curve with a nitrogen flow rate of 120mL/min. The concentration values were converted to their natural logarithm value to help visualize the sensitivity range.

Methanol Conc. [mM]	ln(Methanol Conc.) [mM]	Signal Output [V]
0	0.00	2.81
12.33	2.51	4.81
24.59	3.20	5.74
36.8	3.61	6.24
48.95	3.89	6.57
97.52	4.58	7.12
145.72	4.98	7.37
193.55	5.27	7.47
241.01	5.48	7.55

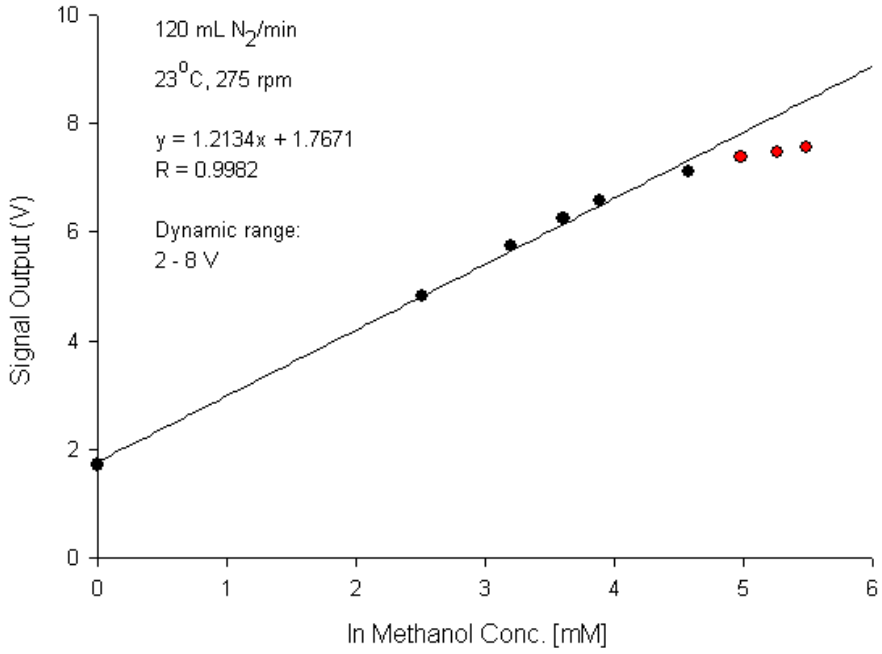


Figure 19. Calibration curve using 120mL/min N_2 flow rate without aeration
Red data points are not part of the linear fit

Figure 20 shows the calibration curve with a nitrogen flow rate of 500mL/min. The concentration values were converted to their natural logarithm value to help visualize the linearity range.

Methanol Conc. [mM]	ln(Methanol Conc.) [mM]	Signal Output [V]
0	0.00	5.69
12.33	2.51	6.85
24.59	3.20	7.25
36.8	3.61	7.4
48.95	3.89	7.52
97.52	4.58	7.68
145.72	4.98	7.77
193.55	5.27	7.81
241.01	5.48	7.86

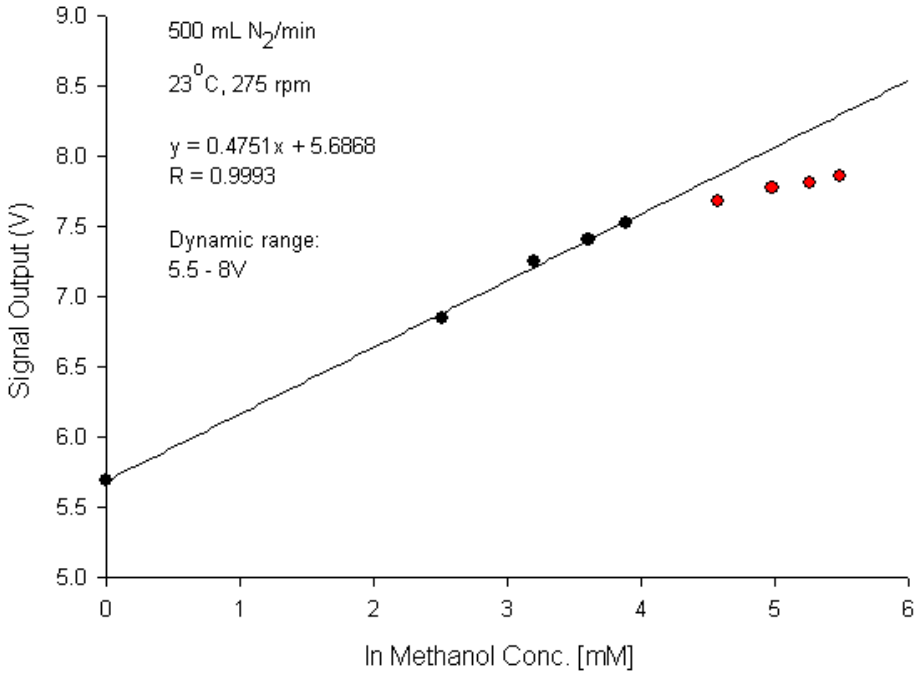


Figure 20. Calibration curve using 500mL/min N_2 flow rate without aeration
Red data points are not part of the linear fit

Note how both curves have a region of methanol concentration that is nonlinear (>100mM). Therefore, the sensitivity of the sensor is best when measuring dissolved methanol concentrations below 100mM. Figure 20 is a representation supplied by

the vendor of the sensitivity loss of the TGS-822 sensor when using different hydrocarbon gas concentrations:

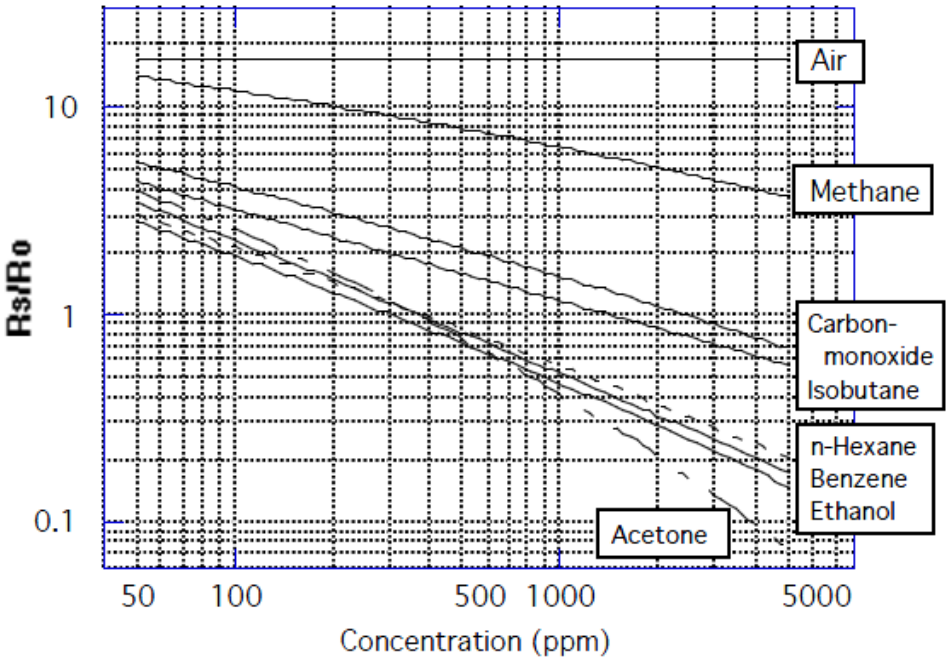


Figure 21. Sensitivity characteristics of the TGS-822 sensor

Rs = Sensor resistance of displayed gases at various concentrations

Ro = Sensor resistance in 300ppm ethanol (Figaro USA)

4.5 Methanol probe response time

Earlier research has shown that during fed-batch fermentations, the silicone tubing sensing method for monitoring dissolved methanol operated independently of fermentation aeration and agitation and that the silicone tubing was not altered by repeated sterilizations (Yano *et al.,* 1976). However, there are other factors that would affect the response time of the methanol probe. If an analogy to a galvanic DO probe is made, the factors affecting probe performance can be summarized as follows:

$$\Delta\, \boldsymbol{Output\ signal} \propto \boldsymbol{A}\left[\frac{\boldsymbol{P}}{\boldsymbol{b}}\right][\boldsymbol{MeOH}]$$

Equation 2. Factors affecting the methanol probe response
A = Silicone tubing area; P = Permeability coefficient of the silicone tubing for methanol
b = tubing wall thickness; [MeOH] = Methanol concentration

Figure 22 demonstrates that there is no change in response time when changing the flowing nitrogen rate as both curve shapes remain practically the same with respect to response time to a step change in methanol concentration of approximately 10 to 12.5 minutes. The mixing time, which is approximately 1.5 minutes, has been subtracted from this calculated response time. In a similar experiment with the 2L bioreactor model, a 150mM pulse at 700rpm (same tip velocity), at 23ºC and 105mL N_2/min, the response time was 12 minutes, which is comparable to the results with this larger probe.

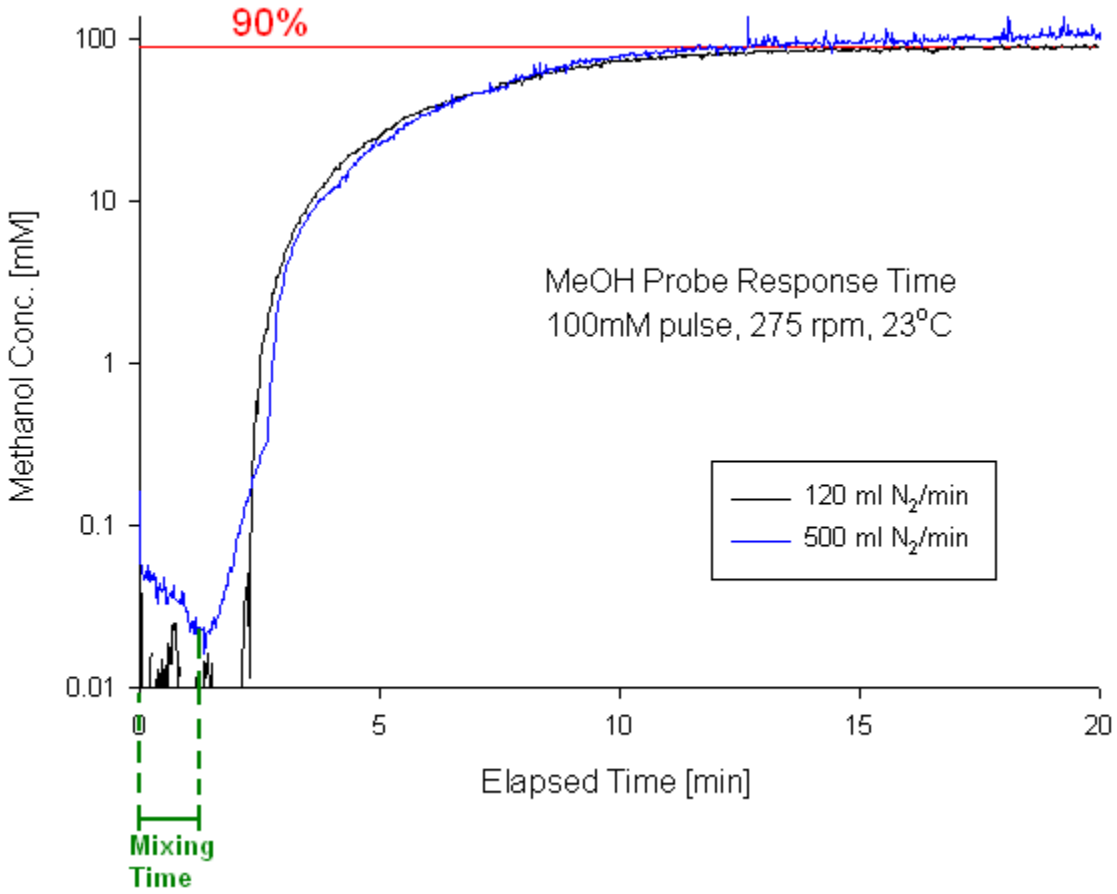


Figure 22. Methanol probe response time to a methanol pulse at two different N_2 flow rates

It is necessary to prove that the probe will respond effectively in high-density fed-batch fermentations with *Pichia pastoris.* It has been observed that *P. pastoris* can reach cell densities of up to 450 g WCW/L in BSM medium supplemented with PTM1 trace salts in a quasi-steady state (Zhang *et al.,* 2011). Therefore, assuming cell concentrations in that range and a biomass yield on methanol of 1.19 g WCW/g MeOH, the calculated methanol uptake rate would be 52.9 g MeOH/L.h.

The optimum methanol concentration for *Pichia pastoris* Mut^+ is <0.5% (<120mM). If a range between 0.2% (50mM) and 1.0% (240mM) is considered as the residual

methanol concentration limits, then 0.5% (120mM) can be established as the lower set point. At a methanol uptake rate of 52.9 g MeOH/L.h, (27.5 mmol MeOH/L·min), the cells would consume the entire 0.5% of dissolved methanol in 4.4 minutes. Therefore, the response time of this methanol probe may need to be further optimized because it may be too slow to be able to sense and control dissolved methanol concentration in high-cell density fermentations. The methanol uptake rate calculation is shown below:

$$\mathrm{MeOH.U.R} = \frac{(0.144\ \mathrm{h^{-1}}) \cdot 450\ \left(\frac{\mathrm{g\ WCW}}{\mathrm{L}}\right)}{\left(1.19\ \frac{\mathrm{g\ WCW}}{\mathrm{g\ MeOH}}\right)}$$

$$\mathrm{MeOH.U.R} = 52.9\ \mathrm{g\ MeOH/L.h}$$

4.6 *Methanol evaporation or stripping rate monitoring*

In addition to the methanol uptake rate, the methanol evaporation or stripping rate is a consideration when calculating the methanol feed rate. Not only do the cells consume the methanol, but some methanol is lost due to evaporation into the head space. The following equation shows the mass balance for methanol:

$$\mathrm{MeOH_{Feed}} = \mathrm{MeOH_X} + \mathrm{MeOH_{Stripped}} + \mathrm{MeOH_{Dissolved}}$$

where the total methanol fed is equal to the sum of the consumed methanol, the stripped methanol and the dissolved methanol in the vessel.

In order to determine the methanol stripping rate, 26 liters of 100mM methanol solution was prepared inside the 30L bioreactor. The solution was agitated at 275rpm and air was sparged at 0.6 vvm during an overnight period at constant temperature (23ºC). The resulting plot of the dissolved methanol probe signal response (Figure 23) allowed the determination of the methanol stripping rate, which was 0.89mmol/L·h. This value is lower than the value determined for the 2L bioreactor model at 50ºC (4.8mmol/L·h) (Hart, 2010). For the *Pichia* systems, the methanol stripping rate value is not significant when compared to the methanol uptake rate value described in the previous section.

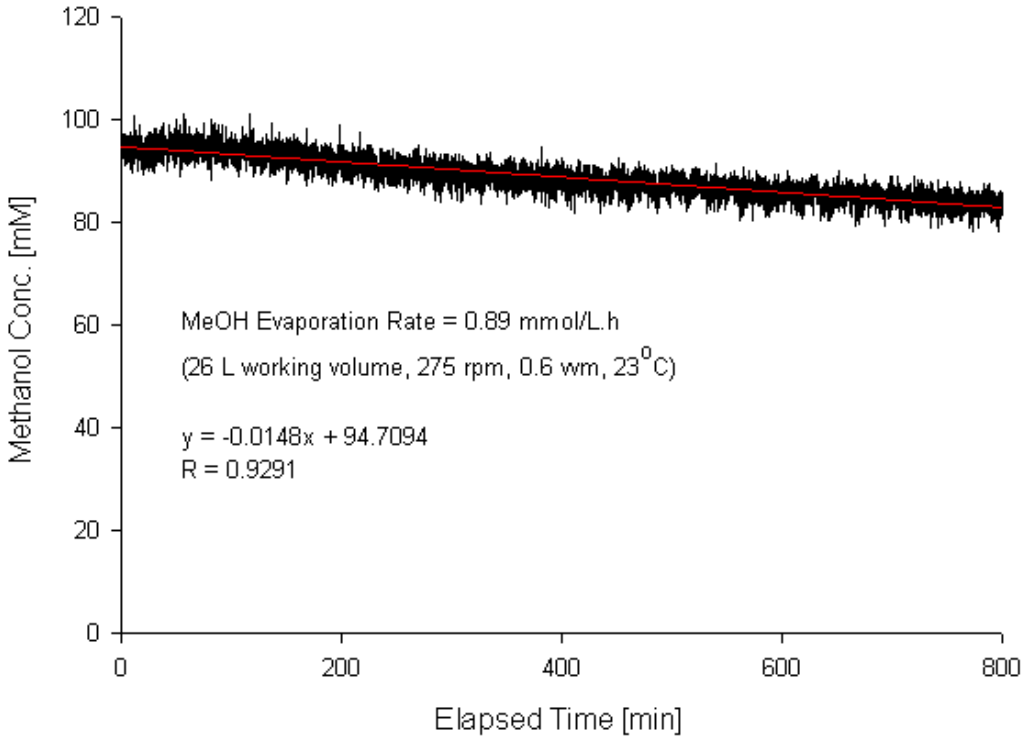


Figure 23. Methanol evaporation rate during aerated and agitated conditions

5 CONCLUSION

An existing 2L bioreactor sensing and control system for monitoring and controlling the dissolved methanol concentration in *Bacillus methanolicus* fermentations was used as a model to implement a similar scheme for a pilot-scale, 30L steam sterilizable bioreactor. The latter system will be used for conducting research in high-density, fed-batch *Pichia pastoris* fermentations for the production of recombinant proteins, by complementing the current inlet and exhaust gas monitoring control panel.

This research demonstrated that technology transfer from one system to another is feasible and the components can be adapted accordingly. A silicone-tubing dissolved methanol probe was scaled-up and was tested with an inexpensive hydrocarbon sensor circuitry and data Labview data acquisition software. The results showed that dissolved methanol concentrations can be quantified but it is necessary with further research to optimize the design of the probe and to adjust operating parameters to obtain a reliable, accurate methanol concentration reading with an effective response time capable of responding to changes in the methanol uptake rate during rapid exponential growth. Finally, the programmed LabVIEW™ Virtual Instrument algorithm along with the data acquisition system will serve as the basis for further I/O signal implementation.

All in all, this study provided deep understanding of the principles of fermentation sensing and control and allowed me to gain experience in the science and engineering of a specific bioprocessing case.

6 FUTURE WORK

All the design, construction and testing of the sensing and control system for the 30L steam sterilizable bioreactor obtained through this research will serve as a starting point for future projects with the 30L bioreactor. Even though a thoughtful design and basic testing was performed, the results showed that it may be necessary to further optimize the probe, test the probe for stability during SIP and to adjust operating parameters in order to obtain reliable, accurate dissolved methanol concentration readings, before implementing a control and methanol feeding strategy to optimize growth of *P. pastoris*

The ideal probe and hydrocarbon sensor reading when a dry and pure carrier gas stream is passed through the sensor should be approximately zero volts when using medium without methanol, which was not the case observed in this study. To expand the sensor's dynamic signal range, it will be necessary to test different nitrogen sources and methods to obtain the purest carrier gas stream. Also, more testing should be performed with different nitrogen flow rates to confirm if this affects the probe response time and determine the effect on signal strength.

Different strategies can be adopted to shorten the probe's response time. The distance from the silicone-tube coil to the sensing element could be a factor affecting response time. Also, a thinner walled silicone-tubing could improve the transfer of methanol from the liquid phase through the silicone tube wall into the carrier gas stream. Moreover, the equilibrium of the liquid and gas phase through the membrane might be affected by process parameters such as temperature, agitation rate, pH, among other factors.

Finally, once the 30L bioreactor sterilization and temperature control features are working properly, the methanol probe should be tested for integrity by performing

steam in place (SIP) cycles with the probe in place. When exposed to sterilization temperatures the silicone tubing integrity and sealing to the stainless steel tubing needs to be studied. Also, with the ability to sterilize the reactor, a chemically defined media could be used to grow *P. pastoris* and to test the probe response to decreasing methanol concentration with temperature, aeration and agitation conditions similar to high-cell density fermentations, as opposed to this preliminary research that used ambient temperature and methanol diluted in water.

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