PRAXIS

A Publication by Bioengineering AG

The Biological Process Development Facility (BPDF) at the University of Nebraska-Lincoln: biopharmaceutical process research; development and manufacturing of candidate vaccines and biotherapeutics derived from recombinant expression systems.

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Bioengineering –
Experience only specialists can have

Between Hickman and Panama. 5.46 p.m.

Why do I live in Nebraska and work at UNL?

The Great Plains of Nebraska have a beauty all their own – waving prairie grasses, rolling hills, wide open skies and spectacular sunsets.

But what makes Nebraska truly special are its people. As you will see, the BPDF staff are some of the best...

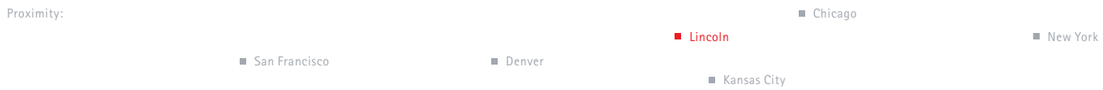
Dr. Michael Meagher
Director of the Biological Process Development Facility (BPDF)
University of Nebraska-Lincoln

41th Street, between Wilber and Clatonia. Strong winds, very cold. 4.13 p.m.


Near Tecumseh. 6.04 p.m.


Unadilla. 4.47 p.m.

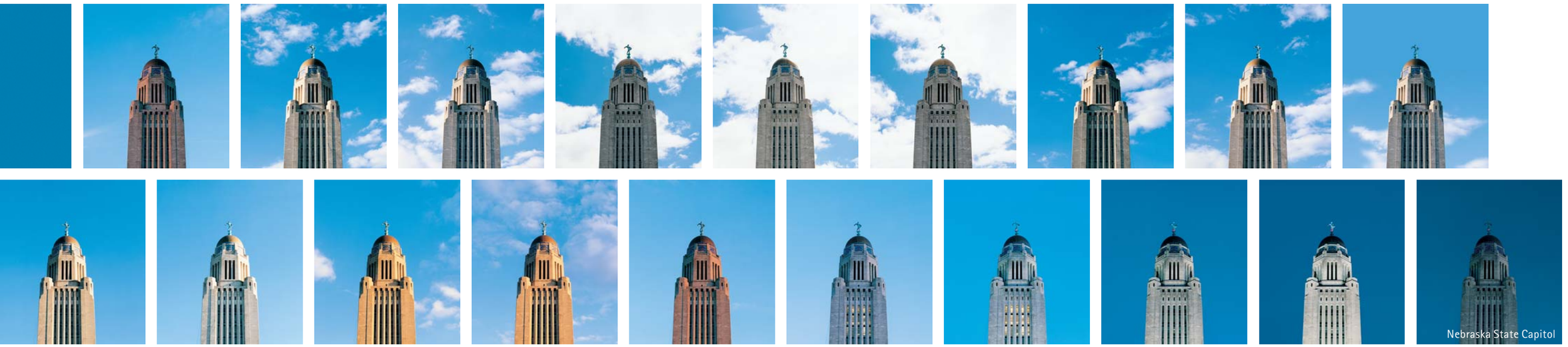

Between Douglas and Sterling. 10.03 a.m.



It was in 1869, less than two years after Nebraska became a state, when the university was chartered and the first students arrived on campus. Some of them, in the words of Nebraska author Willa Cather, "came straight from the cornfields with only a summer's wages in their pockets. They completed the course by really heroic self-sacrifice and there was an atmosphere of endeavor, of expectancy and bright hopefulness about the young college." Nebraska's early settlers recognized the importance of higher education. They took bold steps and built a university based on hope and optimism. They were truly pioneering new frontiers and within a few years, people like Cather, Roscoe Pound, Charles Bessey and James Canfield brought the University of Nebraska to national prominence. By 1895 it was considered one of the four great public universities in the country, along with Michigan, Wisconsin and Berkeley.

Our faculty are passionate about their work, and they are making a tremendous contribution to our state. They are conducting research that will change people's lives, and that is bringing international recognition, as well as important financial benefits, to Nebraska. They are providing classroom and laboratory experiences that attract some of the best students in the state and in the country. The University of Nebraska has a rich history and a bright future. And it is a place of promise for the next generation.

Stated by James B. Milliken, President of the University of Nebraska.



Nebraska State Capitol

6:13 a.m. 6:55 a.m. 7:42 a.m. 9:00 a.m. 9:17 a.m. 9:32 a.m. 10:27 a.m. 10:41 a.m. 11:30 a.m.

1:15 p.m. 2:11 p.m. 3:28 p.m. 3:56 p.m. 4:45 p.m. 7:15 p.m. 8:18 p.m. 9:03 p.m. 2:59 a.m.

Students: All NU 45,477, UNL 21,675.
Faculty: UNL 1,676. Employees: All NU ~11,500, UNL 5,500.

Reseach and external funding:
All NU: $264 million in 2004–05, an increase of 136% since 1995.
UNL: $98.3 million in 2004–05, an increase of 100% since 2000.
$9.98 million grant for Dr. William Velander's work in hemophilia.
$11 million grant for Dr. Ruma Banerjee for redox biology.

Ranking UNL:
Within the top 50 public universities. US News & World Report.
Within the Top 5 places to work in academia by The Scientist magazine.
IANR scientists are in the top 20 in the world for most citations by other authors;
Essential Science Indicators Report, 2004.

Cool faculty:
Ruma Banerjee, Ken Cassman, Dan Claes, Ted Kooser, Marjorie Lou,
Sally Mackenzie, Elizabeth Walter-Shea, Greg Snow, Julie Stone, Jim Van Etten.

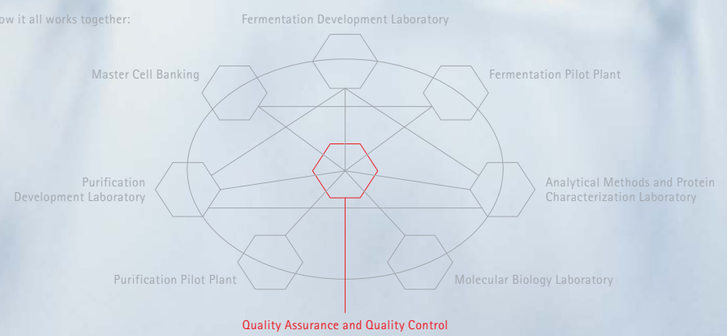
BPDF Managers

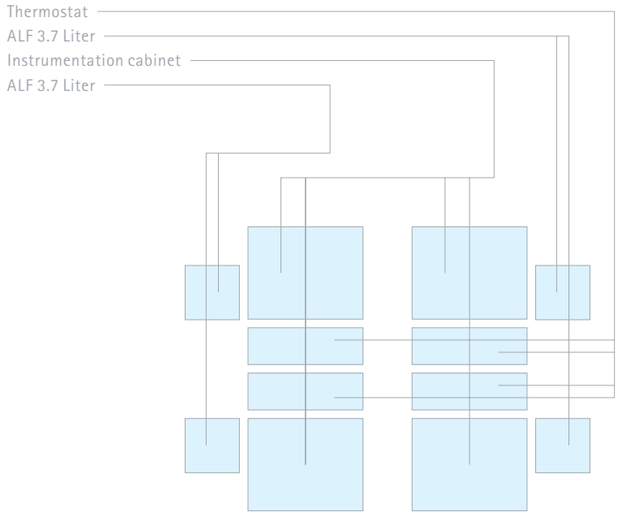
Contents

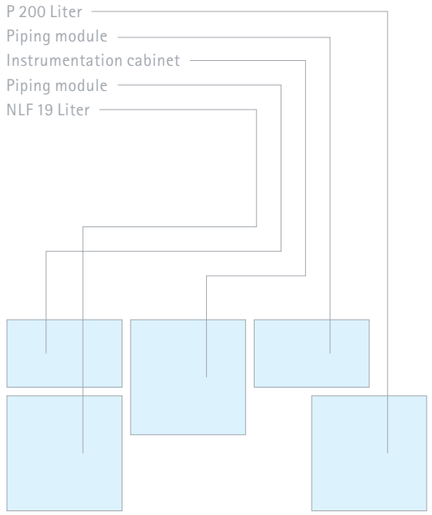
The University of Nebraska-Lincoln Biological Process Development Facility (BPDF) is unique among university-based bioprocessing research programs because of a strong commitment to a Quality Assurance (QA) program. The QA program is an essential component of our cGMP facility and at the BPDF is an integral part of research and development as well. A cGMP-based bioprocessing program must have quality assurance as its central core (see Figure).

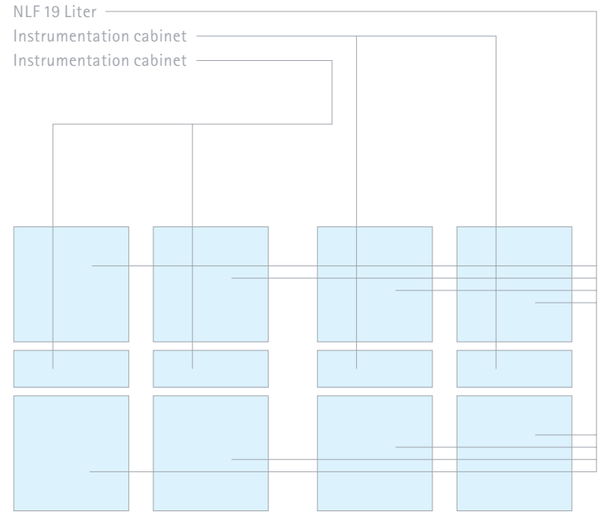
Quality assurance is about documenting all planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality.

BPDF: How it all works together:









The ALF 3.7-liter cell culture reactors are setup for online analysis using a Nova Biomedical BioProfile 400 analyzer. The analyzer regularly samples the reactors for pH, partial pressures of oxygen and carbon dioxide, the ions ammonium, sodium, potassium, the amino acids glutamine and glutamate, and the sugars glucose and lactose. It can be integrated into the fermentor control system using the Batch Expert system described below. The reactors also integrate with our Prima VG mass spectrometer for off gas analysis.

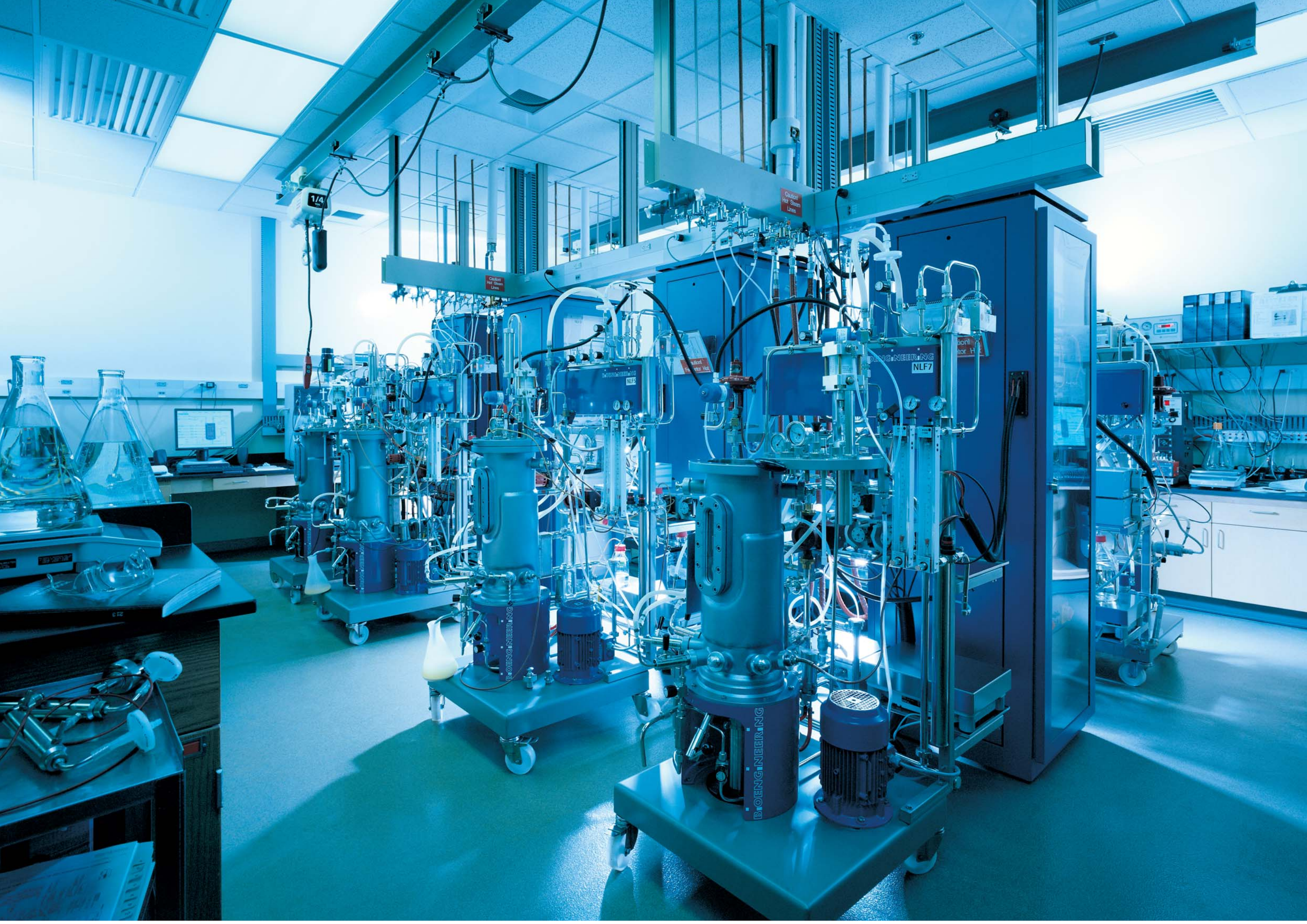
The south half of the Cell Culture Development Laboratory (CCDL) was set up as a non cGMP pilot plant. The pilot plant area contains the 19-liter NLF vessel and the Type P 200-liter vessel. This area of the lab has a built up floor for drainage during cleaning and a sanitizable wall. The 19-liter vessel is set up to act as a pilot vessel or as a seed vessel for the 200 liter. The 200 liter is a unique asset in an academic institution and gives the BPDF a significant advantage.

The Fermentation Development Laboratory (FDL) was built to accommodate eight 19-liter NLF fermentors. All utilities including clean steam, chilled water, air, oxygen, DI water, and uninterruptible power are provided in the overhead carriers. The Bioengineering IFM cabinets are located between two 19-liter NLFs and directly under the service carriers for easy access to power and communication cables. There are separate drains built into the floor that segrate process water and biowaste, which goes to a separate kill system. Kill system drains are located in both the FDL and the CCDL.

Cell Culture Development Laboratory (CCDL). The cell culture lab was uniquely designed for cell culture and specifically set up for our bioengineering equipment. The room was built to operate under positive pressure, using hepafilters. Utilities include clean steam for the reactors, a chilled water supply, and an uninterrupted power system to prevent electrical loss or power surges. Lowered bench height for the reactors allows easy access to the vessels for batch up, cleaning, and operation. A dedicated cold room was attached to the lab with feed lines so that feeds could be refrigerated during runs.

The Fermentation Development Laboratory was also set up to accommodate 8 Bioengineering 19-liter NLF fermentors with dedicated overhead carriers that provide all utilities and power.


BIOENGINEERING


Caution!
Hot Steam
Lines
1/4
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Biological Process Development Facility (BPDF)

The University of Nebraska-Lincoln Biological Process Development Facility (BPDF) is a multidisciplinary "turn-key" facility with currently 49 full-time scientists, engineers, and administrative personnel focused on biopharmaceutical process research, development, and manufacturing of candidate vaccines and biotherapeutics derived from recombinant expression systems. The BPDF is committed to advancing biopharmaceuticals derived from basic research into clinical trials. As researcher identify potential biotherapeutics, they will need access to qualified scientists, engineers and facilities that understand the complexity of bringing a biotherapeutic to Phase I clinical testing. The discovery team should start interfacing with the process development group at the point where microgram to milligram quantities of material are needed for initial testing. The process development group can provide guidance on process-related issues to the discovery scientist that will accelerate the transition from discovery to clinical testing. Examples include choosing the right expression system based on post-translational needs, documenting the expression system for ease of establishing a Master Cell Bank, and establishing essential analytical methods for assessing purity and characterizing the biopharmaceutical.

Educational Mission

As a public university of the State of Nebraska the mission of the BPDF includes serving the needs of the people of the State of Nebraska by educating students, advancing the basic knowledge-base, and acting as a catalyst for economic growth in the State of Nebraska. The academic mission of the BPDF is to:

- provide education in science and engineering focused on bioprocessing, bioengineering and related fields, and to increase the fundamental knowledge basis in bioprocessing and bioengineering through basic and applied research.
- provide a real-world laboratory experience for students in bioprocessing, quality assurance and quality control in a United States Food and Drug Administration (FDA)-compliant facility.

To accomplish this academic mission, a highly-trained staff is required to operate and maintain the facility. The students (post-doctoral, graduate and undergraduate) work with faculty and staff in a mentoring relationship. The post-doctoral and graduate students work on projects that are non-proprietary and results are published in peer-reviewed journals. Undergraduates are provided opportunities to work in the BPDF laboratories at a multiple of levels with the ultimate job of working in the *current* Good Manufacturing Practice (cGMP) Pilot Plant. Only after the students have completed a documented cGMP training program are they qualified to work in the cGMP Pilot Plant.

The BPDF is in the College of Engineering at the University of Nebraska-Lincoln and provides an educational experience for both graduate and undergraduate students in a facility that is in compliance with the United States Food and Drug Administration (FDA) regulations. Students are involved in cutting-edge bioprocess science and engineering in an environment that is under the "umbrella" of a Quality Assurance system. Students receive both academic and GLP/cGMP training and exposure to what is required to bringing safe and efficacious biotherapeutics to the market.

Research Mission

The BPDF has a very strong research and service mission as well:

- Expand the knowledge base in biological process research by fostering faculty interaction through access to state-of-the-art equipment and facilities.
- Provide assistance to government and industry with process research, development, and early manufacturing of new biotherapeutic molecules for human clinical testing.
- To be recognized nationally and internationally as a premier bioprocessing facility.

To accomplish these goals it is essential for the BPDF to be a multidisciplinary facility that can meet all of the requirements to bring a biotherapeutic to clinical trials. This starts with a gene that encodes for a biotherapeutic, developing a production strain to express the product, develop a process suitable for cGMP manufacturing, and produce the biotherapeutic under cGMP for Phase I and II clinical trials. To accomplish this the BPDF has 11 distinct laboratories/units, i.e. Molecular Biology, Fermentation Development, Purification Development, Cell Culture Development, Analytical Methods, Protein Characterization, Quality Control-Chemistry, Quality Control-Microbiology, Master Cell Banking, cGMP Pilot Plant, and Quality Assurance. In addition, there is Information Technology, Maintenance, Business Operations, and Administration to assist with operations. Each of these laboratories/units has a dedicated manager and staff. Central to the BPDF is the Quality Assurance Unit, which oversees all quality programs.

The BPDF (originally called the UNL Fermentation Facility) was founded in 1990 as a joint facility between the UNL Food Processing Center and the UNL Center for Biotechnology to serve the fermentation needs of UNL. Initial capabilities included a 5-liter fermentor, 4 by 1 liter fermentors and a pilot plant with a 60-liter fermentor, high pressure homogenizer for cell disruption, and cross flow membrane filtration system for cell harvesting. These capabilities limited the BPDF to fermentation research and development. Over the last 16 years the BPDF has grown from a 2 person operation focused on fermentation research to a facility with a full-time staff of 49 scientists, engineers, and administrative staff capable of providing everything necessary to bring a biotherapeutic to clinical reality.

This issue of Praxis/Practice presents the Biological Process Development Facility at the University of Nebraska-Lincoln (USA). Research at the BPDF includes understanding the regulation of the Alcohol Oxidase *(AOX)* promotor in the methylotrophic yeast *Pichia pastoris,* the application of neural networking to fermentation control and the integration of molecular biology, fermentation and purification to minimize the effect of endogenous

proteases on the expression and purification of recombinant proteins. The BPDF continues to work with private industry and non-profit institutions worldwide and the United States federal government to bring biotherapeutics to the clinic.
On the following pages is described each of the 11 units/labs of the BPDF and how each is an independent unit and self-sufficient but, as the BPDF, make up a world-class bioprocess research and development center.

Biography of the Director

Dr. Meagher graduated with a Ph. D. in Chemical Engineering from Iowa State University in 1987 and worked for Hoffmann La-Roche (Nutley, NJ) from 1987 to 1989 in downstream processing of recombinant proteins. He accepted a position in June 1989 as an Assistant Professor in the Department of Food Science and Technology at the University of Nebraska-Lincoln. Dr. Meagher's dream was to create a research program which combines the biological sciences, biochemical engineering, and quality assurance with the goal of improving the transition of biotherapeutics from discovery to clinical trials. This vision was derived from his experiences at Hoffmann La-Roche and his love of biochemistry, microbiology and biochemical engineering. The University of Nebraska-Lincoln provided Dr. Meagher with the opportunity to pursue his dream. He has experienced first-hand the positive effect that the biopharmaceutical industry can have on improving life quality. Dr. Meagher combined the University's core missions of research, teaching and service, his love of the biological sciences and engineering, and his wish to have a positive impact on society and started the Biological Process Development Facility in 1990.
Dr. Meagher realized early on that there was a need for facilities that can do process research, develop and scale-up the process, and produce material for Phase I clinical trials. He also realized that a cGMP pilot plant would be a very important part of such a program. Universities have had pilot plants, but they have always been research pilot plants. The challenge of having a pilot plant in universities is the tremendous operating expenses. The primary user of university pilot plants tends to be industry. This brought Dr. Meagher to the conclusion that the only type of pilot plant that would be of benefit to the biopharmaceutical industry was one that met cGMP requirements. This is a significant undertaking for a university because of the commitment to infrastructure, validation of equipment and the facility, and most importantly a quality assurance program. Accomplishing this at the University of Nebraska-Lincoln has taken Dr. Meagher nearly 15 years. Since 1990, the BPDF has grown from 1 technician to a full-time staff of 49 scientists, engineers, technicians, and administrative personnel. In addition, the BPDF provides support for post-docs, graduate students and undergraduate students. In January 2000 Dr. Meagher joined the Department of Chemical and Biomolecular Engineering at UNL and is currently the Donald F. and Mildred T. Othmer Distinguished Professor of Chemical Engineering and Director of the University of Nebraska-Lincoln Biological Process Development Facility.

Molecular Biology Laboratory (MBL)

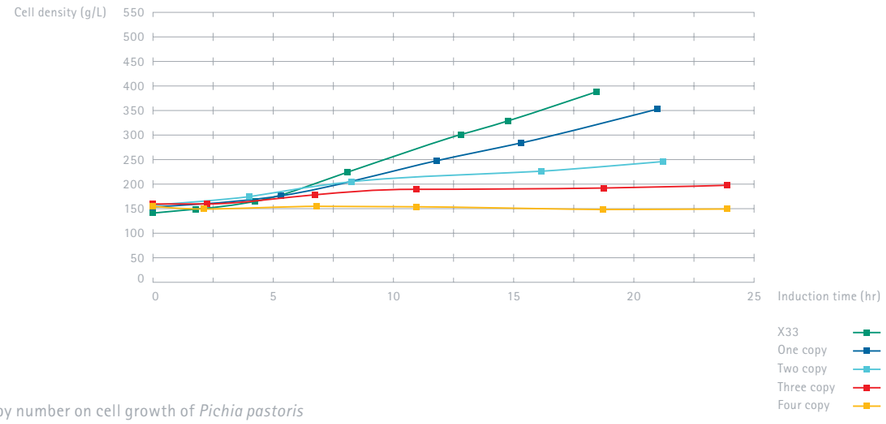
The Molecular Biology Laboratory (MBL) was established to provide the BPDF with capabilities to research, develop, and optimize recombinant expression systems to produce recombinant proteins. The MBL, like the rest of the BPDF, serves clients around the world. The MBL specializes in the molecular biology of *Pichia pastoris,* a eukaryotic expression system that has been successfully used to express a large variety of recombinant proteins. The MBL has also worked with *Escherichia coli, Saccharomyces cerevisiae,* and Chinese hamster ovary (CHO) cells.
The MBL is capable of doing real-time PCR, quantitative PCR (Q-PCR), construction of expression vectors, plasmid transformation (electroporation or chemical transformation), screening and selection of production clones, Northern, Southern and Western analysis, and electric mobility shift assays. The MBL provides the following services:
- Expression vector construction and sequence confirmation.
- Transformation into expression systems and high-throughput screening of clones.
- Optimization of culture conditions for expression at the shake flask level.
- Cell bank production and characterization.

MBL Research

Pichia pastoris, a yeast used as a biological system for the production of recombinant proteins, has gained popularity in the last two decades because of a very strong inducible promoter-system from the gene, alcohol oxidase 1 *(AOX1)*, and the capability to support a scaleable high cell density fermentation process. *Pichia pastoris* is a methylotrophic yeast that grows to very high cell densities in an inexpensive minimal media containing methanol as a sole carbon source. The *Pichia pastoris* system has been used for the production of both industrial and pharmaceutical proteins including enzymes, biotherapeutics, and vaccines against malaria, hookworm, and botulism.

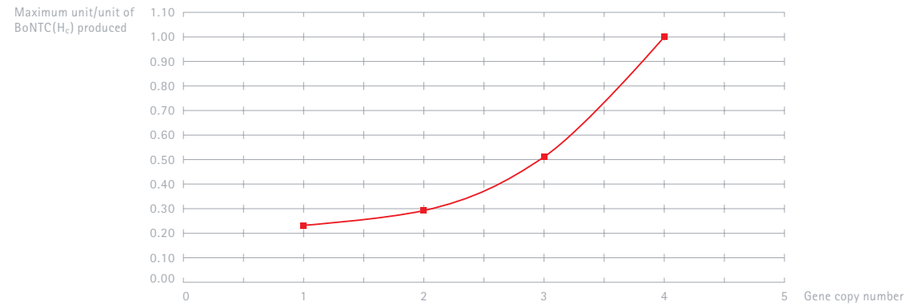
Development of different strength AOX1 promoters

One of the advantages of the *Pichia pastoris* system is that the gene of interest is expressed under the control of the *AOX1* promoter, which is among the most powerful and tightly regulated promoters known. It is induced by methanol and repressed by glucose, glycerol and ethanol. Hence, by-product accumulation, i. e. ethanol and acetate, during protein production halts recombinant protein production and lowers overall productivity.
In order to understand how the *AOX1* promoter is regulated and to ultimately improve the *Pichia pastoris* expression system, we have identified the regulatory regions of the *AOX1* promoter. We employed site-directed mutagenesis and systematic deletion analysis to identify regions of the promoter elements involved in the regulation process. Each fragment was further evaluated for involvement in interaction with regulatory proteins. The *Escherichia coli* lacZ gene was used as a reporter. Electrophoretic mobility shift assay (EMSA) revealed that two regions of the *AOX1* promoter contain sequences for DNA binding proteins. These studies



1

Effect of copy number on cell growth of *Pichia pastoris* strains. X33 strain is wild-type host strain, X33 (pPICZA) host strain with expression vector only.



2

Effect of copy number on rBoNTC(H_c) production.

resulted in a US patent (US patent No. 6699691). Understanding the regulation of the *AOX1* promoter will enable us to design weaker or stronger promoter as needed. We are in the process of identifying, purifying and characterizing *AOX1* promoter DNA binding proteins. By isolating and knocking-out and/or over-expressing regulatory genes we hope to elucidate the molecular mechanisms by which methanol regulates the activity of this promoter. In addition to understanding the basic molecular biology of the promoter, these studies are expected to lead to the development of different strength promoters and *Pichia pastoris* strains that provide optimum protein expression in high cell density fermentations.

Enhancement of secretory capacity of yeast

The amount of recombinant protein produced per cell is well correlated with the copy number of the gene of interest for intracellular protein production in *Pichia pastoris.* However, very high-level expression of secreted proteins saturates the capacity of *Pichia's* secretion system. We are looking for the bottlenecks in the secretion pathway of recombinant proteins by analyzing sub-cellular fractions contained secretary organelles, e.g. endoplasmic reticulum (ER) and Golgi. We and others have shown that co-expression of the endoplasmic reticulum (ER) resident chaperone protein, protein disulfide isomerase (PDI), can improve secretion of entrapped proteins inside the cells. Our research is focused on understanding all aspects of protein secretion from leader sequences, to signal cleaving proteases, to identifying which organelles accumulate recombinant protein. The long-term objective is to customize the expression and secretion of recombinant proteins.

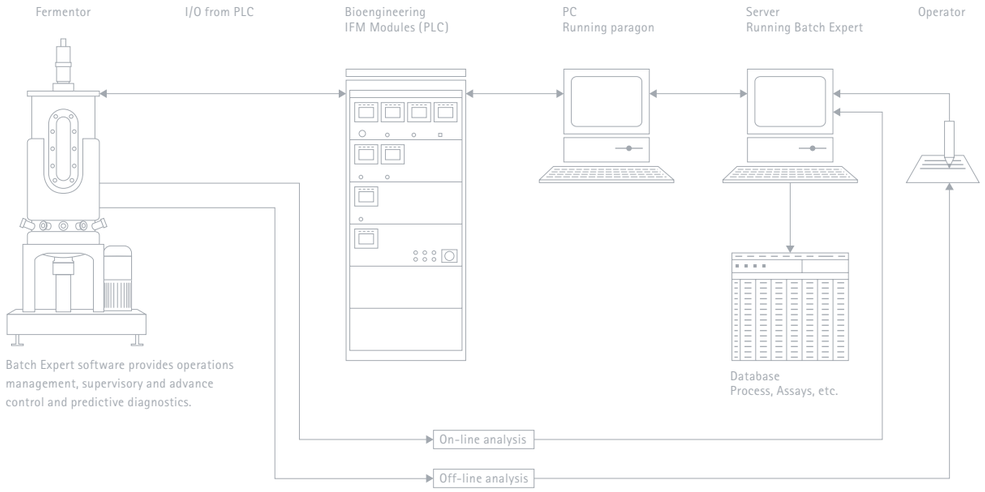
Expression of cytotoxic proteins

Pichia pastoris is a highly effective system for expression of proteins that are toxic to cells. Since the *AOX1* promoter is repressed during growth on alternative carbon sources, i.e. glycerol, toxic protein production does not occur. Once the cells reach the desired cell densities during fermentation, protein production starts with addition of methanol. At this stage, product accumulation continues even though the cells cease to grow due to toxicity of the recombinant protein.

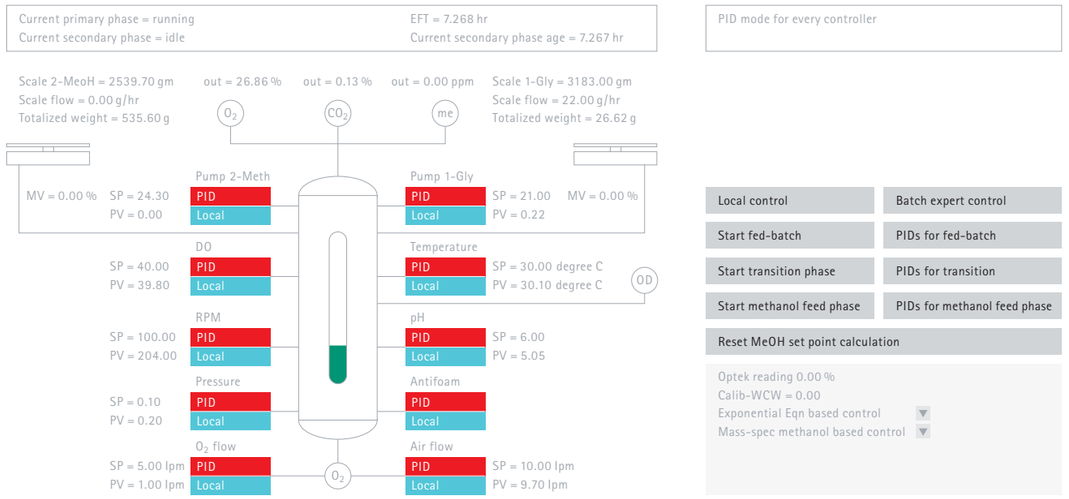
The effect of gene copy number on recombinant protein expression has been evaluated for expression of a vaccine candidate against Botulism toxin serotype C.
Growth rates of *Pichia pastoris* clones expressing one, two, three and four copy of rBoNTC(H_c) have been compared (Figure 1). Increasing copy numbers of rBoNTC(H_c) proportionally reduced methanol utilization of the corresponding strains. In contrast, the wild-type strain, X-33, and X-33 carrying null plasmid (pPICZA) had indistinguishable growth rates on methanol. Although methanol utilization, i.e., growth rate (μ) on methanol, was reduced during induction phase, protein production increased five fold in four-copy clone relative to a single-copy clone (Figure 2).

Our goal at the BPDF is to understand how rBoNTC(H_c) affects the metabolic state of *Pichia pastoris.* Proportionally, this may be a very effective way of decoupling growth rate from heterologous protein production.

Fermentation Development Laboratory (FDL)



3 Integration of Batch Expert with other components of the process (courtesy of ILS).



4 End user interface for NLF 22 type fermentor. NLF 8 equipment group.

Fermentation research at the BPDF is focused on modeling, optimization, control, and metabolic flux analysis of *Pichia pastoris* at high cell density during the production of recombinant proteins. The Fermentation Development Laboratory (FDL) is well equipped with 18 computer-controlled fermentors i.e., ten 5-liter fermentors and 8 highly automated Bioengineering 22-liter NLF fermentors. All 18 fermentors are integrated into a Prima VG mass spectrometer for off-gas analysis. The BPDF uses both dedicated methanol off-gas analyzers and the mass spectrometer to monitor methanol concentration during fermentation. Additional off-line analysis is accomplished with either a YSI 2700 or a Nova Biomedical BioProfile 400 analyzer.

Fermentation Operations

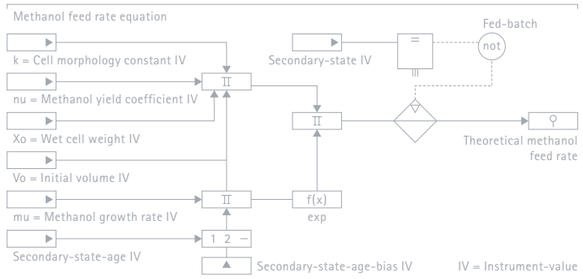
Fermentation activities encompass both academic research and "Fast Track" contractual research. Our client work has included producing recombinant proteins for use as vaccines, active enzymes for medical treatments, and growing micro organism biomass for other applications. Under contract, the FDL has worked with *Pichia pastoris, Saccharomyces cerevisiae,* and *Escherichia coli.*

While initial fermentations are still done in our older 5-liter glass vessel reactors, our best tools are the NLF reactors. The NLF reactors allow us to mimic pilot plant reactors allowing our research to directly transfer into pilot plant reactors. The larger vessels also allow increased production with their higher working volume. This has been critical as the FDL is a cornerstone for all the groups in the BPDF in supplying material for the purification development lab, the analytical methods development lab, and finally for producing material for our clients.

Gensys/Batch Expert Control System

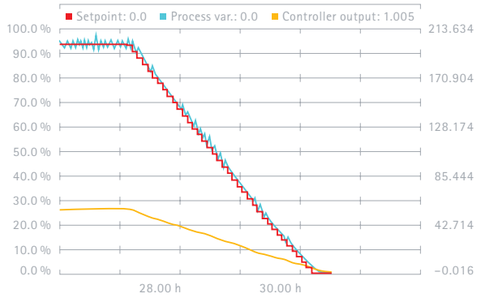
The BPDF is a Gensym University Center of Excellence (COE). Gensym Corporation is a leading provider of adaptable software products for modeling, simulating, and managing business and industrial processes. As a COE, we receive support from Gensym on the following software packages:

- G2, an object-oriented real-time programming platform.
- GDA (G2 Diagnostic Agent), a tool developed in G2 for graphical programming via Information flow diagrams (IFD), makes real-time process calculations and process analysis easy and understandable.
- NOL (NeurOn-Line), a specialized environment developed in G2 for developing and running intelligent operator applications such as fuzzy logic and neural network models in real time while using information flow diagrams (IFD).



5

Information flow diagram to implement deterministic model for substrate feed rate.



6

Control of substrate feed rate profile.

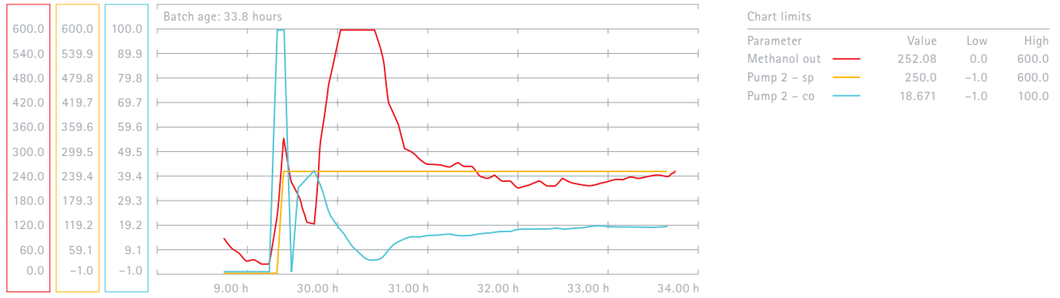


Chart limits

Parameter	Value	Low	High
Methanol out	252.08	0.0	600.0
Pump 2 – sp	250.0	-1.0	600.0
Pump 2 – co	18.671	-1.0	100.0

7

Single batch trend chart showing the control of level of the methanol in the off-gas stream.

The BPDF is a collaborator with Intelligent Laboratory Solutions, Inc. (ILS, Naperville, Illinois), a developer of a software package called Batch Expert that operates under G2 with other integrated packages. The BPDF and ILS have jointly developed a customized software control system to control the 22-liter NLF fermentors.

A schematic of how the control system is set up is presented in Figure 3. The Batch Expert operator interface can be customized to the application as shown in Figure 4. The left side of the operator interface shows the input and output values from the bioreactor, while the right side lists *Pichia pastoris* specific fermentation controls, such as "Start Fed Batch," "Start Transition Phase," "Start Methanol Feed Phase," and "Restart Methanol Setpoint Calculation."
All of these buttons refer to specific steps in the *Pichia pastoris* fermentation process, i.e. "Start Fed Batch" refers to the glycerol feed program for the glycerol fed-batch phase, the "Start Transition Phase" refers to the glycerol and methanol feed program that transitions *Pichia pastoris* from glycerol to methanol, and "Start Methanol Feed Phase," is the feed program during the methanol induction phase, which can be based on a methanol concentration setpoint or a preprogrammed methanol feed rate.

An example of an information flow diagram that is used to determine the methanol feed rate when the "Start Methanol Feed Phase" button is activated is presented in Figure 5, and the graphical data for the glycerol feed is shown in Figure 6.

Finally, Figure 7 shows a trend from Batch Expert of the control of methanol in the liquid phase based on off-gas analysis from the mass spectrophotomer.
Batch Expert is able to readily adapt to incorporating on-line sensors, such as biomass and methanol, and off-gas mass spectrometer. On-line readings and off-line data (e.g. assay results) can be incorporated to real-time calculation and integrated with archived data to generate advanced control algorithms. Besides automatic generation of customized reports, Batch Expert provides simultaneous data access to multiple users. Information can now be delivered from the scientist to the operator through specific recipes combined with an interactive graphical user interface. Deterministic and neural-network-based predictive models are being developed and implemented in real time. Fully automated recipes for desired applications are being developed.
The goal of working with Bioengineering, Gensys, and Intelligent Laboratory Solutions is to develop a software control system that provides the greatest level of flexibility and adaptability so that individual control strategies can be implemented with the objective of optimizing both quality and quantity of the product. Batch Expert has the sophistication required for advanced fermentation research, but can be simplied for process-specific applications in a cGMP application. The G2/Batch Expert system was also selected because it is accepted in industry and, most importantly, there is a well established history of validating the software in an FDA-compliant fashion (21 CFR part 11). The goal at the BPDF is to have Batch Expert controlling both development and cGMP bioreactors. This will provide for a seamless technology transfer into manufacturing.

Pichia Fermentation Research

Pichia pastoris is a methylotrophic yeast capable of growing to very high cell densities (over 400 g wet cell weight/liter) on a defined medium and expressing recombinant proteins in grams/liter quantities. Well defined fermentation control strategies are essential to consistent yield and quality of biotherapeutics and transfer of fermentation processes into cGMP manufacturing. The BPDF was the first to propose an unstructured growth-rate model for optimization of an intracellulary expressed recombinant protein in *Pichia pastoris* (described below). We applied growth-rate models to both intracellular and secreted products and have found the highest yield for most recombinant proteins occurs at a growth rate $^1/_3$ to $^1/_2$ of the maximum growth rate. The advantage of a growth-rate dependent approach is that it is straightforward and readily transferable to a cGMP process.

Fermentation modeling

Fermentation modeling and control, in addition to strain improvement, will have the greatest impact on improving the fermentation process. Modeling is essential to understanding the fundamental behavior of biological processes and is very useful for design, optimization, scale-up, and control. Modeling strategies can be divided into three categories: unstructured models, structured (metabolic) models, and knowledge-based (KB) models such as fuzzy logic, artificial neural network, and expert systems.

Unstructured models

Unstructured modeling, as the name implies, takes a holistic view of the fermentation process and is based on mass balances and kinetic equations. A fermentation process can be described by the following system of first order differential equations:

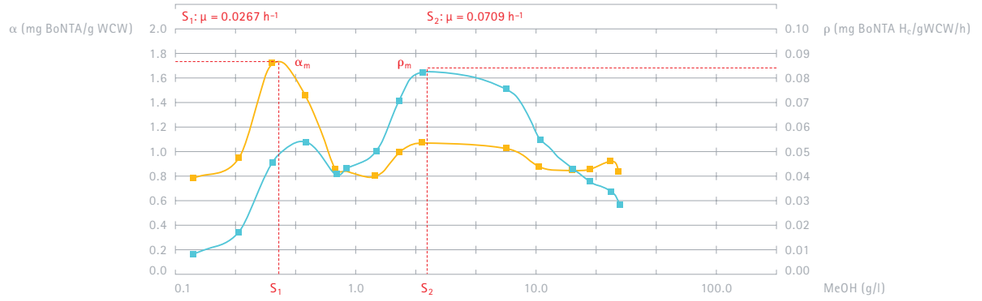
$$\frac{d(XV)}{dt} = X_{in}F_{in} - X_{out}F_{out}\mu(XV) \qquad (1)$$

$$\frac{d(SV)}{dt} = S_{in}F_{in} - S_{out}F_{out} - \nu(XV) \qquad (2)$$

$$\frac{d(PV)}{dt} = P_{in}F_{in} - P_{out}F_{out} + \rho(XV) - \pi(PV) \qquad (3)$$

$$\frac{dV}{dt} = F_{in} - F_{out} \qquad (4)$$

where t is time, X, S, and P respectively are concentration of substrate, biomass, and product in the broth. V is the broth volume, F_{in} is the input substrate feed rate at a concentration X_{in}, S_{in}, P_{in}, and F_{out} output broth flow rate at X_{out}, S_{out}, P_{out}. μ, ν, and ρ respectively are the specific growth, substrate consumption and production rate, and π is the product degradation constant. This system can be used for modeling a batch, fed-batch, or CSTR fermentation process. The BPDF has worked with the United States Army Medical Research Institute of Infectious Disease (USAMRIID) since 1995 on developing processes to produce



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Effect of growth rate on the intracellular expression of BoNTA(H_c).

vaccines candidate protein against the 7 serotypes of the botulinum neurotoxin (BoNT). The vaccines candidates are approximately 50,000 dalton fragments of the C-terminus portion of the heavy chain(H_c). There are 7 distinct serotypes, A-G, requiring 7 distinct recombinant vaccines within a single vial. The BPDF has worked on serotypes A, B, C, E and F and is working on serotypes D and G. Fermentation optimization of the vaccine candidate against serotype A, i.e. BoNTA H_c, was first published by Zhang et al. (2000). Zhang et al. determined the relationship between μ and S, μ and ν, as well as α_m and μ_{crit}:

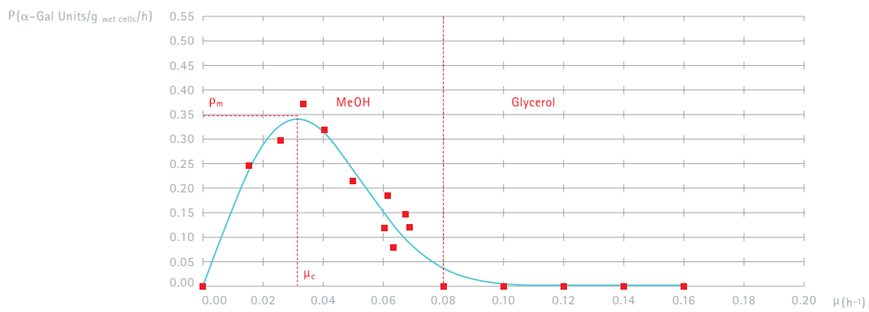
$$\mu = \frac{0.146\, S_{MeOH}}{1.5 + S + S_{MeOH}^2/8.86} \qquad (5)$$

$$\nu_{MeOH} = 0.84\,\mu + 0.0071 \qquad (6)$$

$$\nu_{NH_3} = 0.14\,\mu \qquad (7)$$

$$\alpha_m = 1.72 \text{ mg/g at } \mu_{crit} = 0.0267\ h^{-1} \qquad (8)$$

where α is the BoNTA H_c content in the biomass, mg/g wet cells. This was successfully applied to process design, simulation, and maximizing BoNTA(H_c) production. Figure 8 shows the effect of growth rate (methanol concentration) on the *intracellular* production of BoNTA H_c. The maximum amount of BoNTA(H_c) per unit of cell mass was produced at a growth rate of 0.0267 h^{-1} (= $^1/_3$ maximum growth rate on methanol for this particular construct). A similar unstructured model was applied to the secretion of α-galactosidase from *Pichia pastoris* and (Figure 9) where the optimum production occurred at a growth rate of 0.03 h^{-1}.



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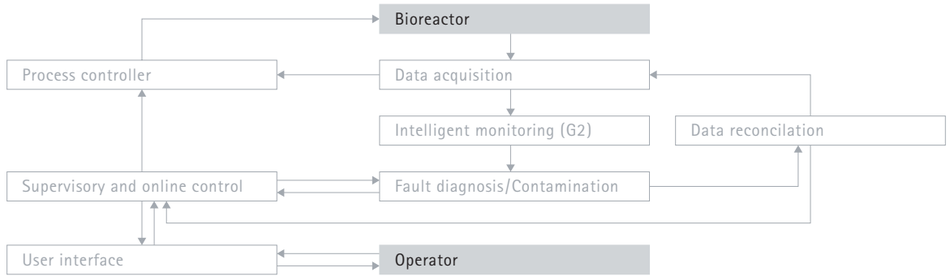
Effect of methanol growth rate on production of secreted α-galactosidase.



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An intelligent bioreactor control configuration.

Structured models

Since unstructured models are based on the assumption that the fraction of a particular metabolite in the cell, for example RNA or DNA, is constant, they do not recognize the complex set of metabolic reactions occurring within the cell. To understand cellular regulation and predict the dynamic behavior of cells subject to changing external conditions, structured models are necessary. Structured models are required for sophisticated control of biological reactors or biological processes.

Structured models can be described by the following system of equations as described by (Williams, 1967), Esener et al. (1982), and Nielsen et al. (1991a; 1991b):

$$\sum_{i=1}^{N} {}_{ji}S_i + \sum_{i=1}^{M} {}_{ji}P_i + \sum_{i=1}^{L} {}_{ji}X_i = 0; \quad j = 1, 2, \ldots, j \qquad (9)$$

$$\frac{dX}{dt} = \Gamma^T r + \Gamma_s^T r_s + \Gamma_p^T r_p - \mu X \qquad (10)$$

$$\frac{dS}{dt} = A^T r + A_s^T r_s + A_p^T r_p - \mu S \qquad (11)$$

$$\frac{dP}{dt} = B^T r + B_s^T r_s + B_p^T r_p - \mu P \qquad (12)$$

$$\mu = \sum_{i=1}^{L} \sum_{j=1}^{J} {}_{ji}r_j \qquad (13)$$

where S_i, P_i and X_i are the ith element of intracellular substrates, products and biomass, respectively. α_{ji}, β_{ji} γ_{ji} are stoichiometric coefficients.
The forward reaction rate for the jth reaction is r_j. X, S and P are concentration vectors for biomass, substrates, and products, respectively. Γ, A and B are stoichiometric matrix for the biotic elements, substrates, and metabolic products, respectively. r is the rate vector for the intracellular reactions (h^{-1}). μ is the specific growth rate for the biomass.

At BPDF, we have developed a structured two-compartment model for *Pichia* growth and protein production using methanol or methanol-glycerol mixed substrates and carbon as an energy source.

Knowledge-based (KB) approaches

Our objective is to apply knowledge-based (KB) approaches to build more accurate simulation and prediction of the process for the purpose of optimization and control. Figure 10 shows the configuration of an intelligent bioreactor control system.

The BPDF Fermentation group is working on the development of soft sensors for prediction of cell mass and other critical variables, such as pH, dissolved oxygen, and recombinant protein production.
The goal is to develop neural networks that can predict critical parameters and serve as a redundant control system.

Relevant fermentation work published by the BPDF.

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Modeling *Pichia pastoris* Growth on Methanol and Optimizing the Production of a Recombinant Protein, the Heavy-Chain Fragment C of Botulinum Neurotoxin, Serotype A.
Biotechnology and Bioengineering. 70(1):1-8, 2000.

Wenhui Zhang, Mehmet Inan, Mike Meagher.
Fermentation Strategies for Recombinant Protein Expression in the Methylotrophic Yeast *Pichia pastoris.*
Biotechnology and Bioprocess Engineering 5(4):275-287, 2000.

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Non-Repressing Carbon Sources for Alcohol Oxidase *(AOX1)* Promoter of *Pichia pastoris.*
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Mehmet Inan, Mike Meagher.
The Effect of Ethanol and Acetate on Protein Expression in *Pichia pastoris.*
Journal of Bioscience and Bioengineering. 92:337-341, 2001.

Wenhui Zhang, Len Smith, Brad Plantz, Vicki Schlegel, Mike Meagher.
Design of Methanol Feed Control in *Pichia pastoris* Fermentation Based Upon a Growth Model.
Biotechnology Progress. 18(6):1392-1399, 2002.

Scott Johnson, Wenhui Zhang, Len Smith, Karen Hywood-Potter, Todd Swanson, Vicki Schlegel, Mike Meagher.
Scale-up of the Fermentation and Purification of the Recombinant Heavy Chain Fragment C of Botulinum Neurotoxin Serotype F Expressed in *Pichia pastoris.*
Protein Expression and Purification. 32(1):1-9, 2003.

Wenhui Zhang, Karen Hywood Potter, Brad Plantz, Vicki Schlegel, Len Smith, Mike Meagher.
Pichia pastoris Fermentation with Mixed-Feeds of Glycerol and Methanol: Growth Kinetics and Production Improvement.
Journal of Industrial Microbiology and Biotechnology. 30(4):210-215, 2003.

Jayanta Sinha, Brad Plantz, Wenhui Zhang, Mark Gouthro, Vicki Schlegel, Chih-Ping Liu, Mike Meagher.
Improved Production of Recombinant Ovine Interferon-t by Mut + Strain of *Pichia pastoris* Using an Optimized Methanol Feed Profile.
Biotechnology Progress. 19(3):794-802, 2003.

Wenhui Zhang, Chih-Ping Liu, Mehmet Inan, Mike Meagher.
Optimization of cell density and dilution rate in *Pichia pastoris* continuous fermentations for production of recombinant proteins.
Journal of Industrial Microbiology and Biotechnology. 31(7):330-4, 2004.

Jayanta Sinha, Brad Plantz, Mehmet Inan, Mike Meagher.
Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris:* Case study with recombinant ovine interferon-tau.
Biotechnology and Bioengineering. 2005 Jan 5;89(1):102-12.

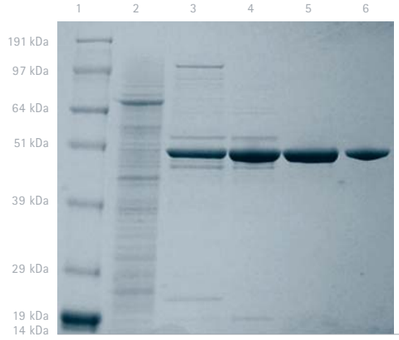
The Purification Development Laboratory (PDL) has the responsibility of purification research and purification process development and is focused primarily on biotherapeutic molecules that are destined for human clinical trials.
This requires an approach that takes into account aspects that are not usually considered at the academic research scale. Purification issues include scalability of the process, technology transfer ability of the process to a cGMP manufacturer, availability and suitability of reagents, supplies, and chromatography resins for cGMP manufacturing, bacterial and endotoxin removal, viral clearance, identification of appropriate hold steps during processing, and stability of the product under different storage conditions.
A lot of these issues are critical to the success of producing cGMP material for human clinical trials.

The PDL is well-equipped for purification research and development. Equipment includes five Applied Biosystems chromatography workstations that are capable of 0 to 100 ml/min flow rates with online measurement of UV absorbance (2 wavelengths), pH and conductivity. Cell disruption capabilities range from bench to pilot scale including an APV Gaulin CD30 homogenizer with an operating pressure of 15,000 psig and a Microfluidic EH-110 microfluidizer with an operating pressure up to 25,000 psig.
Four cross flow membrane filtration systems manufactured by North Carolina SRT are available for use. These units can handle from 0.3 liters to 200 liters of processing volume with membrane areas of 0.1 to 5 m^2. The PDL is also equipped with all of the standard laboratory equipment to support protein purification.

Purification Research

The BPDF classifies purification research into four distinct areas: 1) capture of the protein of interest, 2) purification, 3) polishing, and 4) bulk formulation. Prior to the capture step is the recovery step, which is the separation of soluble (secreted product) from insoluble material, typically cells.
For example, the recombinant botulinum vaccine is expressed intracellularly in *Pichia pastoris,* requiring recovery of the cells, disruption using a high pressure homogenizer or chemical permeabilization, and clarification by either centrifugation or crossflow membrane filtration. The product is then captured from this clarified homogenate.

The capture step has three objectives in the following order of importance; 1) recover as much product as possible in as short a time as possible, 2) generate a product pool that is suitable for chromatography, and 3) remove as many host contaminants as possible. On average, 40% of purification development is spent on the capture step. The quality of the material for capture can vary dramatically as well. In some cases the level of secretion of the product into the supernatant can be so high that the product appears to be over 80% pure



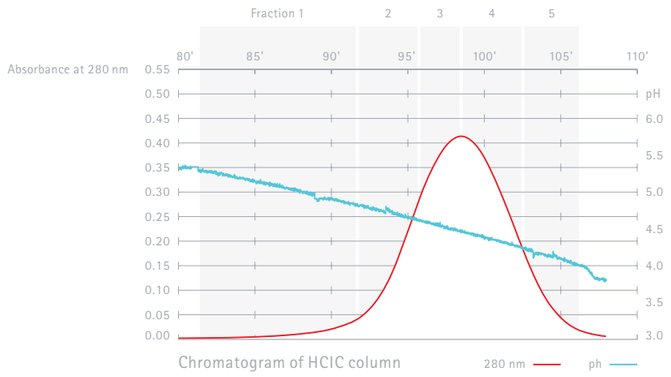
SDS-PAGE of the different product peaks from all the chromatography steps
in the rBoNTE(H_c) purification process with equal total protein load in each lane.

Lane 1: See blue plus 2 molecular weight marker
Lane 2: Cell lysate
Lane 3: Sp sepharose FF product
Lane 4: Q sepharose FF product
Lane 5: SP sepharose HP product
Lane 6: HCIC product

"right out of the fermentor." Capture of this material is very straightforward. In the case of the botulimum vaccines, which are expressed intracellularly and represent only 0.5 to 1.5% of the total soluble protein in the homogenate, the capture step can be very challenging. The PDL successfully used several technologies for the capture step, including fluidized bed chromatography, packed bed chromatography, batch binding chromatography, and crossflow membrane filtration.

The captured material is then purified using standard column chromatography. The objective of purification is to develop a process that removes all host contaminants and in some cases, degradation products. In addition, the process should have a minimal number of buffer exchange steps, which reduces processing time and cost. The final purification step is usually a polishing step and is focused on removing degraded forms of the final product. Because of the biochemical similarities that typically exist between the final products and degraded forms of the product, this step tends to be the most challenging. Chromatographic resin selection and processing conditions are selected to take advantage of such small differences. The polishing step must be supported by good analytical techniques that can distinguish the different species, which sometimes vary by a single amino acid.

The final step in purification research and development is bulk formulation. This is usually an iterative process between the PDL and the formulation group. The challenge is that once the PDL has come up with a process to produce high-quality material, the next question is



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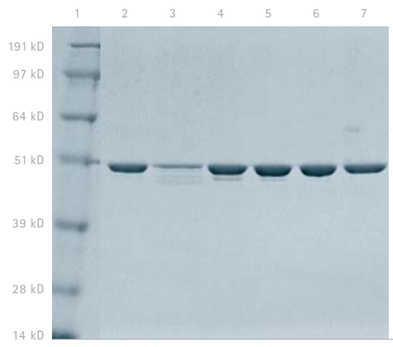
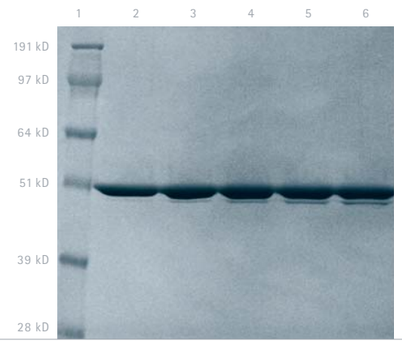
Chromatogram and SDS-PAGE of the HCIC column step showing how the degradation product is removed across the elution of the rBoNTE(H_c) peak.

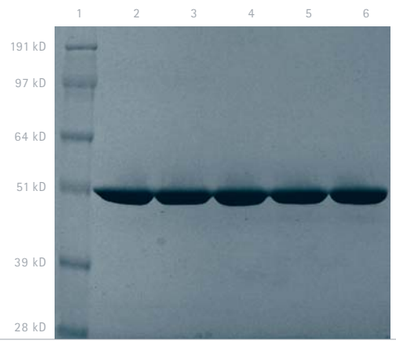


SDS-PAGE of HCIC column step. Degradation product removal across elution of rBoNTE(H_c) peak.
Lane 1: See blue plus 2 MW marker
Lane 2: HCIC load
Lane 3: HCIC fraction 1
Lane 4: HCIC fraction 2
Lane 5: HCIC fraction 3
Lane 6: HCIC fraction 4
Lane 7: HCIC fraction 5



SDS-PAGE of rBoNTE(H_c) final product in 15 mM succinate pH 5.0 at 4°C for 4 days.

Lane 1: See blue plus 2 MW marker
Lane 2: rBoNTE(H_c)-DP-007 Day 0
Lane 3: rBoNTE(H_c)-DP-007 Day 1
Lane 4: rBoNTE(H_c)-DP-007 Day 2
Lane 5: rBoNTE(H_c)-DP-007 Day 3
Lane 6: rBoNTE(H_c)-DP-007 Day 4



SDS-PAGE of rBoNTE(H_c) final product in 15 mM succinate pH 4.0 at 4°C for 4 days.

Lane 1: See blue plus 2 MW marker
Lane 2: rBoNTE(H_c)-DP-008 Day 0
Lane 3: rBoNTE(H_c)-DP-008 Day 1
Lane 4: rBoNTE(H_c)-DP-008 Day 2
Lane 5: rBoNTE(H_c)-DP-008 Day 3
Lane 6: rBoNTE(H_c)-DP-008 Day 4

Stability of rBoNTE(H_c) in buffers, pH 5.0 versus pH 4.0

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how to store the product for later use in stability and formulation studies. The problem requires an iterative solution with the PDL screening pH, conductivity and protein concentration at varying temperatures in an effort to determine a way to store the product for formulation studies. Results from the formulation studies will indicate what conditions are best for storing the product. This information is reported to the PDL, and adjustments are made to the purification process and final storage conditions.

On the previous page there is an example of a process to purify a recombinant protein in the heavy-chain fragment C-terminal domain of the botulinum neurotoxin serotype E (BoNTE H_c) expressed intracellularly in *Pichia pastoris.*
The process requires recovery the cells from the fermentation broth, cell disruption step to homogenize the cells, clarification by centrifugation, and filtration to generate a lysate. Each step with yields is presented in the table on page 39. An SDS-PAGE gel of each step is presented in Figure 11.
Please notice that a distinct BoNTE(H_c) band is not present in the lysate. Western blot was used to visualize the BoNTE(H_c) in the lysate (not shown). The chromatogram of the polishing step for the rBoNTE(H_c) protein is shown in Figure 12, a hydrophobic charged induction chromatography (HCIC) column. The polishing step successfully removed a degradation product from the intact product. Finally, preliminary stability studies were performed on the purified bulk material and it was found that a succinic acid buffer solution with a pH of 4.0 resulted in greater stability than the same buffer at pH 5.0, Figure 13.

Purification of rBoNTE(H_c)[a].
([a]: Purification from 1.3 kg cells wet weight.)

Step	Type	Total Protein mg	BoNTE(H_c) mg	Yield %
			Purity	
Lysate	Clarification	107250	3217	100
			3%	
SP Sepharose FF	Capture	4045	2831	88
			70%	
Q Sepharose FF	Purification	2700	2160	67
			80%	
SP Sepharose SP	Purification	1252	1152	36
			92%	
HCIC	Polishing	780	772	24
			99%	
Diafiltration	Bulking	734	727	22
			99%	

Cell Culture Development Laboratory (CCDL)

The Cell Culture Development Laboratory (CCDL) is the newest addition to the Biological Process Development Facility (BPDF).
The CCDL is composed of two different laboratory rooms. The first lab is for cell culture transformation, and growth of cultures in T-flasks and small spinner flasks. This lab is equipped with three biosafety cabinets, CO_2 incubators, inverted microscope, and ample bench space. The second laboratory is the cell culture bioreactor room which permits complete scale-up from 0.250 liters to 200 liters. This lab contains two computer-controlled DasGip systems capable of running 8 independent spinner flasks with on-line-D.O. and pH and four 4-liter Bioengineering ALF bioreactors, 19-liter Bioengineering NLF and a 200-liter Bioengineering pilot skid. All Bioengineering bioreactors are computer-controlled.

Research in the CCDL has focused on the expression of antibodies in Chinese hamster ovary (CHO) cells and media optimization for the production of antibodies in CHO cells.

The cell culture lab was uniquely designed for cell culture and specifically set up for our bioengineering equipment. The room was built to operate under positive pressure, using hepafilters. Utilities include clean steam for the reactors, a chilled water supply, and an uninterrupted power system to prevent electrical loss or power surges. The cabinetry was laid out to accommodate the ALF reactors. Lowered bench height for the reactors allows easy access to the vessels for batch up, cleaning, and operation. Raised portions of the tabletop were set for the placement of scales and pumps for our automated feed control. Cabinets were set up for securing feed bottles, and some for placement of computer CPUs in the cabinets with separate UPS power outlets and data ports where they are protected from liquids in the lab. A dedicated cold room was attached to the lab with feed lines so that feeds could be refrigerated during runs.

The ALF 3.7-liter cell culture reactors are set up for online analysis using a Nova Biomedical BioProfile 400 analyzer. The analyzer regularly samples the reactors for pH, partial pressures of oxygen and carbon dioxide, the ions ammonium, sodium, potassium, the amino acids glutamine and glutamate, and the sugars glucose and lactose. Such a large array of assays allows for careful attention to operations. The analyzer is capable of integration into the fermentor control system using the Batch Expert system described above.
As well as the analyzer, the reactors also integrate with our Prima VG massspectrometer for off-gas analysis. The ALF units are used for research, as well as for the development of scaleable protocols.

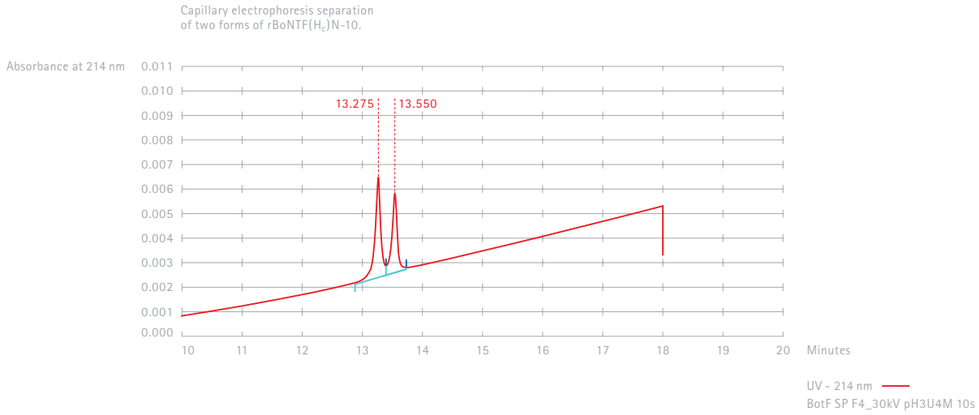
The north half of the cell culture was setup as a non-cGMP pilot plant. The pilot plant area contains the 19-liter NLF vessel, and the Type P 200-liter vessel. This area of the lab has a built-up floor for drainage during cleaning and a sanitizable wall. The 19-liter vessel is set up to act as a pilot vessel or as a seed vessel for the 200 liter. The 200 liter is a unique asset in an academic institution and gives the BPDF an advantage.

Analytical Methods Laboratory (AML)

The Analytical Methods Laboratory (AML) in the BPDF engages in applied research, developing analytical methods to monitor the quality, quantity, stability, and purity of recombinant proteins. These assay methods often do not exist for projects entering the BPDF. Specific, accurate, and reproducible analytical methods are critical to enable process development of fermentation and purification. Analytical methods are required by the FDA to move promising therapeutics or vaccines into human clinical trials. Most projects brought to the BPDF involve recombinant proteins that are candidate molecules to become either a human therapeutic or a vaccine. Analytical methods move these projects forward. The AML was founded in 2003. It has produced 20 analytical methods and continues to produce more. Most of them were transferred into the BPDF-Quality Control Chemistry Laboratory for routine analytical use. Some are transfered to an outside facility for use in commercial production.

Analytical Research

The AML primarily develops analytical methods for molecules of interest where the methods do not exist. Methods development usually occurs in parallel with process development. As a result, "reference" standard material of the molecule of interest may be derived from fermentation and purification processes that are unfinished. This can result in synergies between the process development laboratories and the AML. Example: The recombinant protein rBoNTF(H_c)N-10 is a candidate molecule for a multivalent biodefense vaccine against the neurotoxins of *Clostridia botulinum.* The rBoNTF(H_c)N-10 protein is expressed intracellularly in a *Pichia pastoris* production strain that contains multiple copies of the gene of interest. The fermentation process is a high-yield process producing rBoNTF(H_c)N-10 in the grams of protein per kg wet cell mass range. During purification development, samples were given to the AML for analysis by methods we were developing. This material was thought to be >95% pure by other methods. Capillary zonal electrophoresis (CZE) showed an unexpected result (Figure 14). There were two peaks present in an approximate 60%:40% ratio. CZE separates on the basis of difference in charge on the protein. This CZE method would separate positively charged proteins, with more positively charged proteins giving earlier peaks. This result was confirmed by analytical cation exchange chromatography (another separation based on number of positive charges), replicated on the preparative scale by the Purification Development Laboratory, and verified by both CZE and analytical cation exchange (data not shown). Both peaks were detected in a Western blot using an anti-rBoNTF(H_c)N-10 polyclonal antibody (data not shown).
Preparative fractions containing mainly the two different materials were subsequently purified by reverse-phase HPLC on the analytical scale and analyzed by direct infusion electrospray mass spectrometry to determine the intact masses of the proteins. The early peak in Figure 14, which corresponds to a more positively charged molecule, had a mass of 49,083.6 mass units. The late peak in Figure 14, which corresponds to a less positively charged molecule, had a mass of 49,124.7 mass units. Each of these measurements has an error of



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An electropherogram of the two forms of rBoNTF(H_c)N-10. The capillary electrophoresis was performed on a Beckman PACE MDQ system with a UV detector monitoring 214 nm with a data collection rate of four per second. The electrolyte buffer was 50 mM sodium citrate, 4 M urea at pH 3.0. Electrophoresis was conducted at a constant voltage of 30 kV for 18 minutes. The sample was applied to a neutral coated 30 centimeter by 50 micrometer inner diameter capillary with a 10 second electrokinetic injection.

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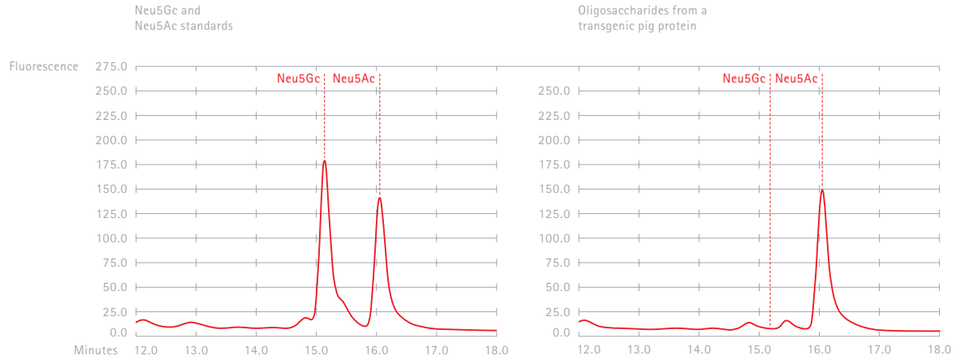
Each of the forms of rBoNTF(H_c)N-10 is shown as rectangle with different shadings. The rectangle represents a scaled linear model of the amino acid sequence of the protein. Ca. 46 % of the amino acid sequence was detected by peptide mapping experiments. The remaining 54 % was not detected in these experiments. Sites of detected chemical modification are shown as shaded lines where the specific modification is indicated by the color of the line.

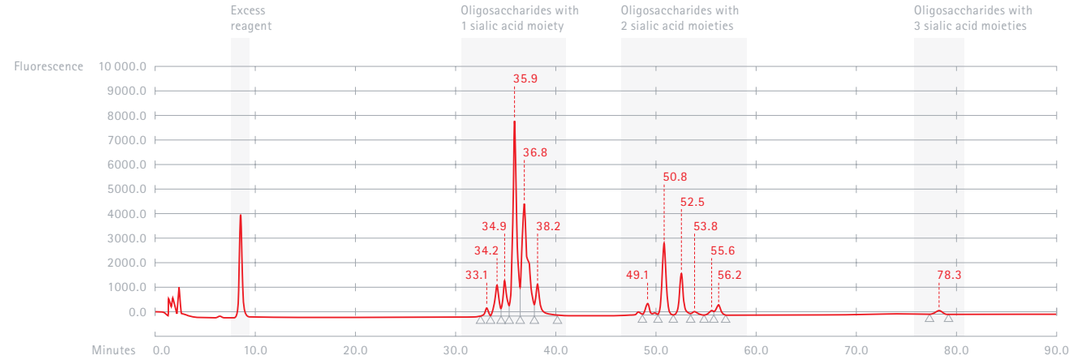
at least ±1 mass unit. The difference of the two masses is 41.1. By theoretical calculation, a modification of a protein by chemically attaching an acetyl group to the amino terminal residue of the protein (acetylation) would result in a mass difference of 42 mass units. This modification would also remove a positive charge from the modified protein. Our measurements are consistent with this modification occurring. We sought to confirm our hypothesis that a portion of the rBoNTF(H_c)N-10 protein was acetylated at the amino terminus by peptide mapping, which is a type of analysis where an enzyme called a protease is used to cut the protein into numerous pieces (peptides). These pieces are then analyzed by reverse-phase high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS). This allows in-depth analysis of peptides and can be used to confirm chemical modifications of individual amino acids in the peptide. The peptide mapping experiment on the late peak (Figure 14) demonstrated that the amino-terminal peptide was completely acetylated. The peptide mapping experiment on the early peak (Figure 14) showed an amino-terminal peptide that was unacetylated. These results taken together confirm the hypothesis that the difference between the two peaks is due to a partially acetylated protein. Additional peptide mapping analysis data for the acetylated and unacetylated forms of rBoNTF(H_c)N-10 show that there are four to seven sites of deamidation of the amino acids asparagine and glutamine that occur specifically in the acetylated form. The chemical process of deamidation results in the creation of a single negative charge from a uncharged species. The net effect on the entire molecule is to decrease the net positive charge on the whole molecule. A representative model of the differences between the acetylated and unacetylated forms of rBoNTF(H_c)N-10 is shown in Figure 15. This data is currently being confirmed. If correct, it explains the differences detected by both cation exchange chromatography and CZE are due to a difference of five to eight net positive charges on the rBoNTF(H_c)N-10 molecules. Having identified the differences between the peaks, we sought to explain why the differential modification is occurring. While deamidation is a chemical process that typically takes place with storage of proteins, this does not explain why only the sites in the acetylated protein are deamidated. If this were due to conditions the proteins were exposed to, it would be reasonable to expect that during the fermentation, acetylated and unacetylated forms of the protein should see the same environments and modification chemistries. Since this does not appear to be the case (Figure 15), a likely explanation is that the acetylated form is processed differently by the yeast than the unacetylated form. Our current operating hypothesis is that either the copy number of genes and/or the fermentation conditions produce so much protein that the expression host cannot completely modify all of the protein. We have started to investigate these possibilities by first creating an analytical cation exchange HPLC assay that is capable of separating the acetylated and unacetylated forms of rBoNTF(H_c)N-10 from clarified cell lysates. Initial evaluations of fermentations using production strains containing various copy numbers of rBoNTF(H_c)N-10 genes suggest the ratio of acetylated to unacetylated is relatively fixed with respect to gene copy number. Additional attempts are underway in the Fermentation Development Laboratory to see if extreme fermentation conditions can affect the ratio or amounts of either protein form significantly.

Protein Characterization Laboratory (PCL)



16

Use of normal phase HPLC (NP-HPLC) to determine if a recombinant glycoprotein contains the sialic acids N-glycolylneuraminic acid (Neu5Gc) or N-acetylneuraminic acid (Neu5Ac). All human proteins contain only Neu5Ac. Neu5Gc is considered to be antigenic in humans, and so it is important that recombinant glycoproteins be carefully characterized.



17

Normal-phase HPLC profiling of N-linked oligosaccharides from a recombinant glycoprotein produced in a transgenic animal bioreactor. Oligosaccharides are separated based on the number of sialic acid moieties present.

The Protein Characterization Lab (PCL) is engaged in research that can be described as *"targeted functional proteomics."* We are looking specifically at how structure affects the function of recombinant proteins that are being produced by a genetically modified organism, be it a transgenic animal, a CHO cell line, a yeast cell line, or bacterial cell line. Many of the therapeutic proteins being developed today are complex in their structure and often contain numerous *post-translational modifications* (PTMs) made to the amino acid side chains. In the proteins we are working with right now, the following PTMs are present: N-linked glycosylation, O-linked glycosylation, phosphorylation, sulfation, γ-carboxylation of glutamate, disulfide bond formation, and proteolytic processing. A complicating factor in the production of recombinant proteins is that there are often *rate limitations* in these PTMs, and so a recombinant protein will often be produced as a mixture of isoforms that vary in the nature and extent of a particular PTM.

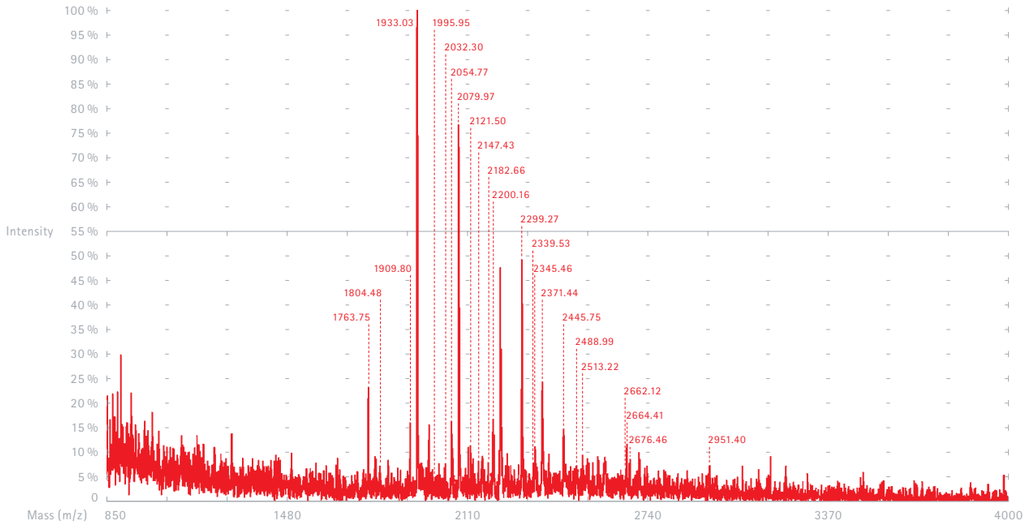
Additionally, this list of modifications becomes even more extensive when one considers the kinds of structural changes that can occur during harvesting, purification, formulation, and storage of a protein – such as deamidation, oxidation, proteolytic degradation, and denaturation.

All of these modifications have the potential to significantly impact a protein's therapeutic effectiveness. Thus, it is our goal to characterize the structure of these isoforms so that structure can be related to *in vivo* function and pharmacokinetic properties. Once we know what structural isoforms exist, we can then work with Molecular Biology, Fermentation Development, Purification Development, Analytical Methods Development, and Quality Control to re-engineer the cell line's metabolism, adjust fermentation conditions, refine purification processing, and develop new analytical methods so that the best possible therapeutic is produced. This work requires expertise in protein purification and analytical biochemistry. The PCL routinely uses FPLC, HPLC, SDS PAGE, isoelectric focusing, immunoassays, mass spectrometry, and enzyme assays to characterize the proteins.

Characterization Research

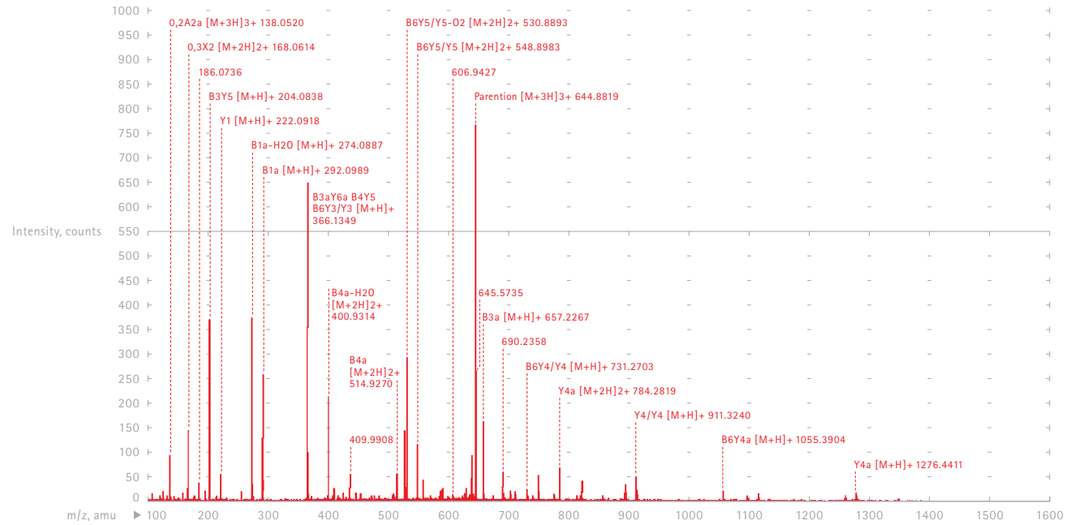
One focus area of research is the characterization of the structure of N-linked oligosaccharides on recombinant glycoproteins. The PCL is one of the few laboratories in the midwestern United States that has the expertise to use HPLC and mass spectrometry to determine oligosaccharide structures and compositions.

We are using HPLC to perform monosaccharide composition analysis and to determine whether the oligosaccharides contain the sialic acids N-glycolylneuraminic acid (Neu5Gc) or N-acetylneuraminic acid (Neu5Ac). The sialic acid identity is important, as human proteins contain only Neu5Ac, and glycoproteins with Neu5Gc are considered to be antigenic. Figure 16 shows the analysis of a recombinant glycoprotein produced in a transgenic pig



18

MALDI-TOF MS profile of acidic N-linked oligosaccharides of a glycoprotein.

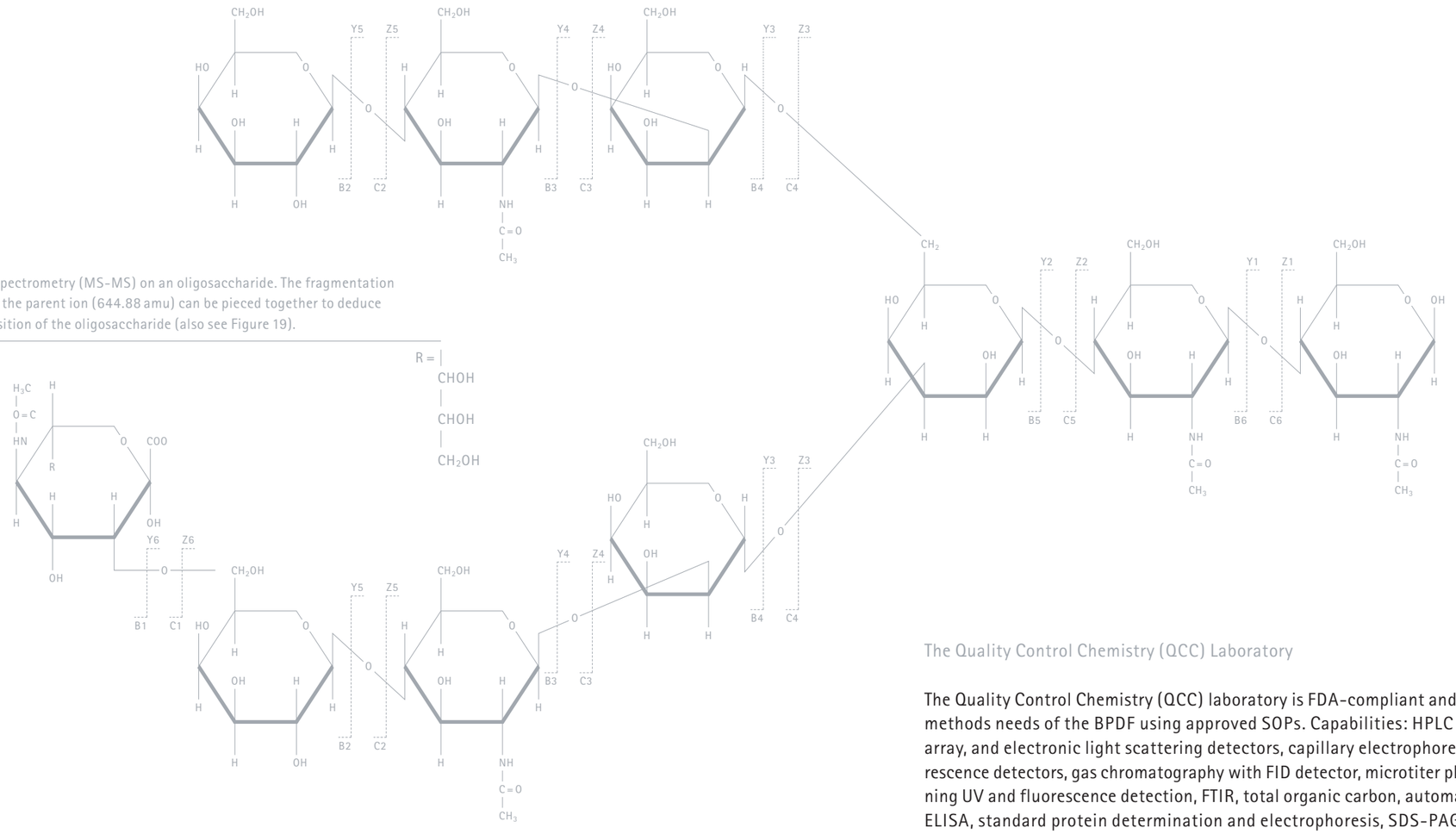


19

Results of tandem mass spectrometry (MS-MS) on an oligosaccharide. The fragmentation pattern that results from the parent ion (644.88 amu) can be pieced together to deduce the structure and composition of the oligosaccharide (also see Figure 20).

bioreactor. We have found that the transgenic pig does not produce oligosaccharides having Neu5Gc in this particular recombinant glycoprotein. We also use HPLC to profile and obtain a fingerprint of the oligosaccharide structures in glycoproteins. Shown in Figure 17 is the fingerprint of a recombinant protein with only two potential N-linked glycosylation sites. This technique separates oligosaccharides on the basis of how many sialic acid moieties are present. Elution times are very reproducible, and by integrating peak areas the consistency of the glycosylation status of a recombinant protein can be quantitatively determined. As can be seen, there is a significant complexity to these glycoproteins, with at least 13 different oligosaccharides being found on these two glycosylation sites.

Once we know the monosaccharide composition and have a good idea of the complexity of the oligosaccharide profile of a particular glycoprotein, we use mass spectrometry to determine oligosaccharide structures. MALDI-TOF MS is used to obtain a quick profile of what neutral and acidic oligosaccharides are present in a glycoprotein (Figure 18). Then the details of the structures are determined by using electrospray ionization tandem mass spectrometry (ESI-MS-MS). Oligosaccharides are fragmented in the collision cell of the mass spectrometer, and the fragment ions are used to deduce the oligosaccharide structure and composition, as shown in Figure 19 and 20.
The PCL is creating its own database and library of oligosaccharide structures found in the glycoproteins we are analyzing, and we are developing methods to facilitate the analysis of MS-MS spectra.



Results of tandem mass spectrometry (MS-MS) on an oligosaccharide. The fragmentation pattern that results from the parent ion (644.88 amu) can be pieced together to deduce the structure and composition of the oligosaccharide (also see Figure 19).

The Quality Control Chemistry (QCC) Laboratory

The Quality Control Chemistry (QCC) laboratory is FDA-compliant and serves the analytical methods needs of the BPDF using approved SOPs. Capabilities: HPLC with UV, photodiode array, and electronic light scattering detectors, capillary electrophoresis with UV and fluorescence detectors, gas chromatography with FID detector, microtiter plate reader with scanning UV and fluorescence detection, FTIR, total organic carbon, automated LAL (endotoxin), ELISA, standard protein determination and electrophoresis, SDS-PAGE, Western blot, IEF, 2-D SDS PAGE. The QCC also oversees 5 validated stability chambers; 2 chambers capable of 5 °C to 60 °C with humidity control, 2 chambers capable of –20 °C to 80 °C with humidity control, 1 chamber capable of 25 °C to 80 °C without humidity control and a –80 °C chamber.

Quality Control Microbiology (QCM) Laboratory

The Quality Control Microbiology (QCM) laboratory provides microbiological services to the BPDF, such as environmental monitoring of the cGMP facilities and microbiological release tests for master cell banks and working cell banks. It is equipped with standard equipment for performing routine microbiology and is capable of doing sterility tests. The QCM has a Qualcom Riboprinter, an automated system for identifying bacteria and some yeasts. All equipment is validated and all methods follow approved SOPs.

Master Cell Banking Suite, cGMP Pilot Plant

Master Cell Banking Suite

The Master Cell Banking Suite (MCBS) is designed to produce a validated Master Cell Bank (MCB) and the Working Cell Bank (WCB) for bacteria, yeast and mammalian cell lines. All cell banking operations are performed in an isolator that is fully validated and can be sterilized-in-place using vaporized hydrogen peroxide (VHP). The isolators system is composed of two distinct units. The main isolator is designed to perform all cell banking operations, i.e. harvesting and distribution of cells into cryovials, while the second and smaller isolator, referred to as an isolette, is a fully controlled and sterilizable incubator for growing up the cells prior to distribution. The isolette is docked to the main isolator during the VHP cycle for sterilization. The isolator system, which includes the main isolator, isolette, and the utility panel for the isolette, is built by Cone Craft Inc. (Dallas, Texas USA).

cGMP Pilot Plant

The BPDF is designing and building a new cGMP pilot plant that will accommodate bacteria and yeast at the 300-liter scale and mammalian systems at the 100-liter scale. The new cGMP pilot plant will be capable of producing lyophilized vialed product. Previously, the BPDF had a cGMP pilot plant that operated at the 500-liter scale and produced purified bulk product.

Quality Assurance

The Quality Assurance Unit is the foundation for documentation of all development activities in the BPDF, successful technology transfer for commercial production, and cGMP production of phase I/II clinical material.

The BPDF has a 5-person Quality Assurance Unit (QAU) that is responsible for the BPDF quality system. The QAU oversees the compliance with Good Manufacturing Practices (GMPs) by the Quality Control Chemistry and Quality Control Microbiology Laboratories and pilot plants. QAU also oversees Good Laboratory Practices (GLPs) compliance by the Fermentation Development Laboratory (FDL), Purification Development Laboratory (PDL), Molecular Biology Laboratory (MBL), Analytical Methods Laboratory (AML), and Protein Characterization Laboratory (PCL) of the BPDF.

Activities include:

- Management of the documentation system including standard operating procedures, GMP production batch records, raw material acquisition, and release oversight, and any necessary technology transfer documents.
- Collaboration with BPDF staff to generate the necessary documents to successfully transfer/produce the product.
- Auditing of in-house process development and production.
- Identification and surveillance of outsourced services.
- Oversight of calibration and maintenance of equipment, validation of major utility systems and their revalidation programs.
- IQ, OQ and PQ of all new equipment related to GMP work.
- Assistance to establish either a process that can be transferred into a GMP facility or production of GMP material for clinical trials.

The BPDF recently installed a Cerity Electronic Content Management (ECM) System®, an electronic data management system to collect, organize, warehouse, index, and safely archive all structured and unstructured electronic records from raw data and laboratory reports into compliance records, putting the information the BPDF needs to improve operations in one, convenient, easily accessible location. Cerity ECM® provides a secure, Part 11-compliant, centralized electronic library for any and all electronic data files of any size. Not only can machine-readable instrument data files be stored, but also images, multimedia files, presentations, human-readable word processing and Adobe PDF documents, spreadsheets, and hundreds of other formats. Cerity ECM® automatically extracts searchable metadata from each of the files using ECM® SmartFilters.

Using a Web-based Virtual Private Network (VPN), one is able to search any records of interest using an integrated quick or advanced query and view the files in the browser without the need for data-generating applications for most file types.

Ardis Barthuli

Scott Johnson

Dr. Michael M. Meagher

When not saving the world by solving bioprocessing problems, Dr. Meagher plays competitive golf (or completes his wife's "honey-do" list before playing competitive golf).

Chad Andersen

Teresa Andersen

Rick Barent

Made in Peru.

Hugo E. Angeles

Makes mean pots of chilli.

Sheila M. Bart

Ardis Barthuli

Loves livin' downtown.

Paul L. Bates

Jill K. Becker

Raghavendra Bemgal

Samantha Bogus

Youngest member at BPDF.

Ronnie Brown

Kossivi Dantey

Jon Campbell

Michael P. Dux

More than expected.

Sarah Fanders

Recently married.

Sandra Franks

Mojtaba Fatemi

"Well-behaved women seldom make history."

Mark A. Gouthro

Kathy Glenn

Janet Greenwald

John Harms

On a mission to find the perfect chocolate chip cookie.

Jay Harner

William Heidal

Jicai Huang

Mehmet Inan

Vilay P. Jain

Scott Johnson

1 loving wife, 4 kids, 1 dog (and 1 TV).

Chris Kearns

Roger Ladd

Tarian Mammedov

Michelle Mathiesen

Kevin A. McCulloch

Legal, Regulatory, and Language Consultant for the BPDF.

Mark Neyens

The glass is always half full (until the kids knock it off the table).

Andrew Plambeck

Is the ultimate expert in farming.

Bob Sealock

Laura K. Smoyer

Jared Solomon

Amber Swanson

Shin Taoka

A small but a fine hybrid of American Biochemistry and Japanese Material Science.

Galina Yaskevich

Mehtap Yilmaz

Eilleen McCulloch

Michael M. Meagher

Raghavendra S. Philkana

Sreenivasula R. Ramiredoy

Jayanta Sinha

Nick Steffen

S. Todd Swanson

Kevin VanCott

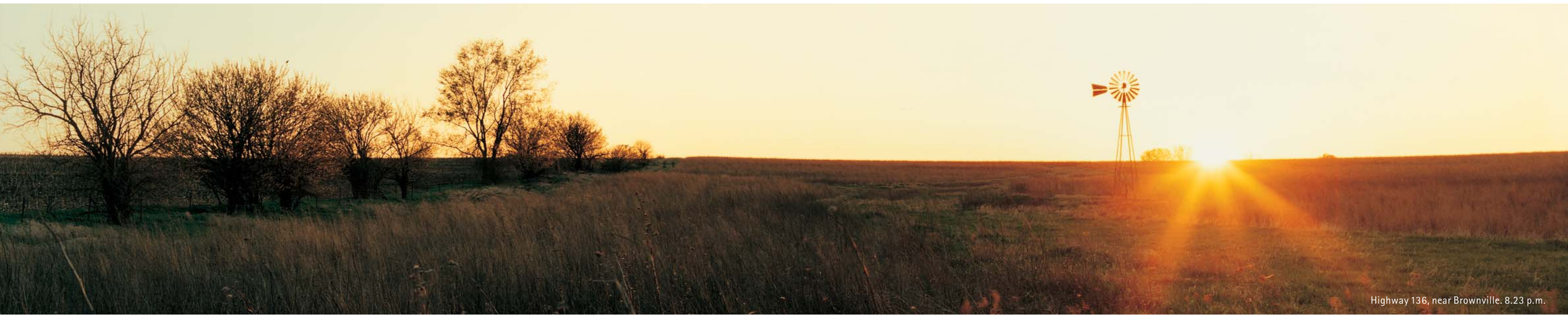
49
enthusiasts at the BPDF

The biopharmaceutical "industry" is a highly competitive field including large pharmaceutical and biotechnology companies, start-up biotech companies, federal agencies and academic researchers. They all must deal with the same issues of how to transition a biotherapeutic from discovery into phase I clinical trials in a fast, safe and cost-effective manner. The research mission of the BPDF is to advance the science and engineering of producing a biotherapeutic and scaling-up the process for clinical manufacturing, with research programs range from molecular biology to fermentation scale-up at the 300-liter scale. This makes the BPDF unique among university research institutes. And this success is only possible with the enormous knowldege, dedication and passion of each individual contributing to our mission.

Jacques Poisson
B.Sc, Marine Biology
University of Dalhousie
2001–2004

Mr. Poisson uses this fishbowl only when he gives interviews, or when he desires to enjoy the panoramic view. Normally he resides in a luxurious aquarium.

Nebraska State Capitol. UNL Dormitories. 4.46 p.m.



Highway 136, near Brownville. 8.23 p.m.

Noises: Wind and various birds.

A bunch of squirrels.

3 prairie dogs.

Windmills, used for pumping water stations on remote farms.

Prairie grass.

A flock of sandhill cranes, hardly visible.

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