Welcome

*ISPE Boston Area Chapter Presents:*

Process Scale-up & Technology Transfer

"Beyond E. coli and CHO:

Case Studies in Alternative Host Platforms"

HOST CELLS for Approved Recombinant Therapeutics in the US

- First recombinant therapeutic - 1982 Humulin (Lilly) - *E. coli*
- First mammalian - 1987 TPA (Genentech) - CHO
- First 10 years 1982-1991:
  - 12 microbial - *E. coli* or yeast and
  - 2 mammalian - CHO
- First 30 years 1982-2011:
  - 50+ microbial - *E. coli* or yeast and
  - 50+ mammalian - CHO, BHK (2), mMCMHC (8+), HEK/human (3)
- First human - 2001 Xigris (Lilly) - HEK, and 2 since then:
  - 2006 Elaprase (Shire) - human cell
  - 2010 VPRIV (Shire) - human fibroblast cell

*Escherichia coli, Chinese Hamster Ovary, Baby Hampster Kidney fibroblasts, Human Embryonic Kidney, murine Myeloma or Hybridoma*

*Statistics from Bioprocess International*
A *Pseudomonas fluorescens* fermentation process for quality recombinant protein production

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Outline

- Pfenex background
- *Pseudomonas fluorescens* as an alternative expression system
- Background on properly folded protein expression
- Pfenex process development approach
- Safety and regulatory considerations
- *P. fluorescens* fermentation process
- Process transfer
Pfenex Overview

- Located in San Diego, California
- 30 employees
- 25,000 square feet of laboratory and product development space housing full capabilities for process development and non-cGMP protein production
- Core technology: *Pseudomonas fluorescens* expression platform

Businesses:
- Strain engineering and process development services
- Product Development
  - Subunit vaccines andbiosimilars
- Reagent Proteins
  - Messenger proteins
  - Enzymes
  - Vaccine components

Established Expression Systems for Pharmaceutical Proteins

*E. coli*
- Non-glycosylated recombinant proteins
  - Growth hormones, insulin
  - Antibody fragments

*Yeast* (*Saccharomyces cerevisiae, Pichia pastoris*)
- Limited complex (non)-glycosylated recombinant proteins
  - Growth hormones, insulin
  - Vaccines

*Mammalian cell culture (e.g. CHO)*
- Complex glycosylated recombinant proteins
  - Monoclonal antibodies
Pseudomonas fluorescens as an Alternative Microbial Expression Platform

Similarities to E. coli
- Simple molecular cloning
- Short development timeline
- Good scalability
- Short cultivation time (24-48 hours)
- Simple defined media
- High titer (insoluble) inclusion body expression with difficult refolding

Differences from E. coli
- Ability to screen high number of production strains with similar amount of effort
- Secretion to the periplasm (supernatant)
- Soluble, active protein product, no refolding needed
- Less effort in downstream processing
- Correct N-terminus
- Titers @ g/L level
- Most advance product in Phase III clinical trial

Pseudomonas fluorescens Background

History of industrial use
- Produced insecticidal proteins for application as biopesticides for >20 years (Mycogen)
- High cell density fermentation at >100,000 liters scale
- Produced a GRAS food processing enzyme at >10,000 liters scale (Dow)
- Expression of biopharmaceutical proteins/biologics (Dow/Pfenex) transferred to various cGMP facilities and subsequently produced at >1,000 liters scale

Characteristics of Pseudomonas fluorescens
- Non-pathogenic Gram-negative aerobic bacterium
- Does not accumulate inhibitory acids
- Growth in a soluble defined mineral salts medium without the need for animal derived products or organic nitrogen
- Amenable to genetic manipulations
  - Genome sequenced and annotated
  - Genetic and recombinant gene expression tools developed
  - Periplasmic expression support proper N-terminus amino acid and disulfide bond formation → soluble expression of active proteins
Importance of Proper Protein Folding

Proper protein folding needed to:
- achieve native structure or conformation to ensure full activity and stability
- avoid unwanted immune response
- prevent aggregation

Inducible Gene Expression → Properly Folded Protein

Proper protein folding more likely to occur in periplasm due to the presence of machinery for disulfide bond formation.
Purification Process Considerations

<table>
<thead>
<tr>
<th>Insoluble inclusion body</th>
<th>Soluble expressed protein</th>
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<tbody>
<tr>
<td>- More unit operations</td>
<td>- Fewer unit operations</td>
</tr>
<tr>
<td>- Lower purification yields</td>
<td>- High purification yields</td>
</tr>
<tr>
<td>- Longer process</td>
<td>- Simpler process</td>
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</tbody>
</table>

- Fermentation
- Centrifugation
- Cell lysis
- Inclusion body recovery
- Solubilization & refold
- Chromatography 1
- Chromatography 2
- Chromatography 3

- Fermentation
- Periplasmic release
- Centrifugation
- Chromatography 1
- Chromatography 2
- Chromatography 3

★ Pfenex preferred

The Pfenex Integrated Approach
Optimal parallel process development for biopharmaceutical protein production - statistical design and data-driven

- Rapid Strain Construction & Screening
- Fermentation Scouting and Optimization
- Primary recovery
- Purification
- Characterization and analysis

Soluble properly folded protein expression
The Pfenex Strain Toolbox:  
The Next Generation for Bacterial Strain Engineering

Plasmid library
- 2 plasmids
- 4+ promoters
- 3 ribosome binding sites
- 25+ secretion leaders
- 45+ chaperone/disulfide bond isomerase overexpression plasmids
- 130+ protease clean deletion mutants, multiple deletions

Host strain library

Thousands of unique combinations are seamlessly integrated to enable strain engineering for optimal protein production in *Pseudomonas fluorescens*.

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High-Throughput Strain Development & Process Analytical

- Analyze all strains by HTP SDS-CGE and/or BLI; select strains by Western blot, LC-MS
- Replicate cultures grown, expressive analysis
- Electroporate and grow transformants on selection media
- Biochemy Interferometry, Function/Filter
- Microchip SDS-CGE, Ther
- Western blot, Quality

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Rapid Fermentation Development

- Minibioreactors and parallel bioreactors are integral to Pfenex’s rapid fermentation development process.
- Minibioreactors (Micro-24) and scalable high cell density process in 2 L glass or 20 L stainless steel bioreactors.
- Multiple strains evaluated in multiple fermentation conditions in DoE.
- Minibioreactor process can be predictably scaled.
- Strain and process finalists scaled to higher cell densities in 2 L or 20 L bioreactors.
- Production strain finalized and fermentation process further developed if needed.

Rapid Primary Recovery Evaluation

- Standard Cell Lysis
  - Homogenization
- Solids Separation
  - Continuous centrifugation
  - Depth filtration units
  - TFF

Periplasmic Release Toolbox
- Continuous osmotic shock process
- Heat treatment
  - controlled temperature and time exposure
- Chemical treatment
- Solvent extraction
  - use of glycol ethers
  - phase-splitting to reduce volume

Screen at small scale

Optimize at bench scale

Evaluate at pilot scale

- Microfiltration plates or small breakers
- Use of statistical DoEs
- JMP for design and analysis
- Assess step yield and purity
- Assess impurity profile
Chromatography Development

- Lead protein strain engineering and delivery of purified protein for proof of concept studies

Project Examples and Timelines

- Lead protein strain engineering and delivery of purified protein for proof of concept studies

- Process development and transfer
Safety and Regulatory Considerations

Clearance of P. fluorescens host contaminants
• Demonstrated clearance of P. fluorescens host contaminants to acceptable levels for injected drugs by standard purification processes
  • Host cell proteins (HCP)
  • Nucleic acids
  • Endotoxins – lipopolysaccharide (LPS) components of outer cell membrane of Gram-negative bacteria

Assurance of clearance
• Development of ELISA for quantitation of P. fluorescens HCP
  • Generation and characterization of P. fluorescens HCP specific serum
  • Assay development and qualification
  • ELISA kit now commercially available
• Verification of applicability of standard assays for P. fluorescens nucleic acids
• Verification of applicability of standard assays for P. fluorescens endotoxin
  • P. fluorescens LPS was purified and determined to be assayable using standard LAL assays
  • rabbit pyrogenicity tests demonstrated equivalent response with reference LPS from E. coli

P. fluorescens Fermentation Process
• Simple defined mineral salts medium with glycerol and ammonia as carbon and nitrogen sources
  • Recipe designed to ensure excess nutrients to support high cell density
  • No animal derived materials or antibiotics
• Fed-batch high cell density fermentation process
  • Cell densities >200 OD (>100g per liter dry cell weight or >2x 10^11 cells/mL)
  • Minimal inhibitory acid production
  • High specific protein expression
  • 2 days or less
• Two-phase production process
  • Growth – biomass generation
  • Production – target protein expression
• Engineering considerations
  • Oxygen transfer rate of ~ 300 mmol/L/hr
  • Heat transfer of ~ 40 kcal/L/hr
Full Fermentation Process

- Seed stages – culture growth phase only
- Production stage – culture growth and production phase
- Considerations for the seed stages:
  1. Inoculum size
     - too small → long lag in culture growth
     - too big → more seed stages needed
     - 2-10% ideal
  2. Phase of seed culture for transfer
     - too early → low initial cell density
     - too late → lag in culture growth
     - mid-exponential or vigorous growth phase ideal

Elements of a Production Fermentor

Sterility
- Sterile entries and exits
  - Additions ports for inoculum, feeds
  - Sterile air/gas inlet
  - Ports for essential sensors

Engineering considerations
- Gas exchange and mixing
  - Aeration rates – air, oxygen supplementation
  - Sparger types
  - Motor power
  - Impeller types, size and numbers
  - Baffles
- Heat exchange through jacket or coils
  - Heating capability during sterilization
  - Cooling capability

Considerations for *P. fluorescens*
- Oxygen transfer rate of ~ 300 mmol/L/hr
- Heat transfer of ~ 40 kcal/L/hr
Typical Process Transfer

- Documentation of 20 L fermentation (and purification) process and associated analytical methods
  - Detailed process description
  - Process flow diagram
  - Sample plan
  - Bill of materials
  - Equipment list
  - Batch production records
  - Analytical protocols e.g. HCP ELISA
- Transfer of process documents
- Onsite training of plant personnel at Pfenex
  - Execute a 20 L fermentation process
  - Compile and transfer of batch production records along with appropriate analytical results
- Transfer of Research Cell Bank
- Shake-down run(s) in scale-down bioreactor at CMO pilot plant
  - Remote or on-site support from Pfenex personnel
- Preparations and shake-down run(s) in production bioreactor at CMO
  - Remote or on-site support from Pfenex personnel

Case Study: Vaccine Protein Fermentation

Nine fermentation parameters investigated in µ-24 minibioreactors

- Two factors have key effects on antigen production
- Optimal operating ranges defined

Improved antigen expression and quality confirmed in scalable 1 L
Scale-up and Transfer to Single-use Disposable Bioreactor

- Scale-up run in XDR-50 Prototype 2
- Growth rates identical, final cell density >200 OD
- Antigen expression can be improved in XDR process but 3MM doses already exceeded in a single batch
- Antigen relatively pure after two columns

Considerations for Successful Process Transfer

- Raw materials from plant qualified vendors
  - Test fermentations to ensure equivalent performance as Pfenex specified vendors
  - Glycerol example – chemically synthesized vs. plant derived
  - Antifoam
- Preparation of sterile mineral salts medium and feeds
  - Batch, continuous or filter sterilization
- Oxygen transfer capability to support high cell density *P. fluorescens* culture
  - Sparger/agitator design
  - Air with/without oxygen supplementation
- Heat exchange capability
  - Cooling fluid type, temperature and flow rates
  - Jacket, coils, etc.
- Control of glycerol feed delivery
  - Control loop based on on-line signal(s) specific to the plant bioreactor
- Extra care needed to ensure sterility of culture in the absence of antibiotics
- Detailed process understanding by plant staff achieved after a few shake-down runs
Acknowledgements

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- Pat Lucy – Business Development
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Jeff Guertin et al. at Xcellerex

Abbreviations

BLI: biolayer interferometry (binding assay)
CMO: contract manufacturing organization
DNA: deoxyribonucleic acid
DoE: Design of Experiments
HCP: host cell protein
HTP: high throughput
JMP: statistical design of experiment software package
LAL: limulus amebocyte lysate assay for endotoxin or LPS
LC: Liquid chromatography
LPS: lipopolysaccharide
MALDI: matrix-assisted laser desorption/ionization, a soft ionization technique used in mass spectrometry
MS: mass spectrometry
OD: optical density
QTOF: quadrupole time of flight mass spectrometer
RP-HPLC: reverse phase high-performance liquid chromatography
SDS-CGE: sodium dodecyl sulfate capillary gel electrophoresis
SEC: size exclusion chromatography
TFF: tangential flow filtration
Expression and Purification of Recombinant Proteins Using the PER.C6® Human Cells

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Why Mammalian?

• Mammalian cells
  • Mammalian cells dominate the field for manufacture of complex proteins (glycosylated) and monoclonal antibodies,
  • Since 1957, CHO was freely available and initially favored because of its robust growth and relatively high yields

• Microbial cells
  • Lack the machinery for proper folding and post translational modification of complex proteins, critical for pharmaceutical performance

• Yeast cells
  • Post-translational modifications differ from mammalian systems

• Others
  • Transgenics, plant systems, and microalgae are still in development

Why Human?

- Accurate human post-translational modifications
  - Species-specific differences of protein glycan structures can have implications for function, clearance, and safety
- No possibility of non-human contaminants in biological products used for human therapy

Why Percivia?

- The PER.C6® cell line was rationally designed for pharmaceutical manufacturing
  - Its creation, characterization, and safety testing are documented in a Biologics Master File submitted to the FDA
- Low cost of goods
  - Percivia has developed an efficient and high yielding protein production platform based on the PER.C6® cell line
- FDA-accepted expression system
  - Approximately 40 clinical trials have been initiated with no adverse effects reported
The Genesis of the HEK293 Human Cell Line

Expression plasmid containing Ad5 E1A and E1B genes

Human embryonic kidney cells

HEK293

The Genesis of the PER.C6® Human Cell Line

Expression plasmid containing Ad5 E1A and E1B genes

Human primary embryonic retinal cells

PER.C6®

The E1 gene with precisely known genetic boundaries was taken from Ad5 and used to transfet primary human retinal cells

HEK293
Safety Testing

- Viral/Bacterial Testing
  - Sterility
  - Mycoplasma
  - In vitro and in vivo adventitious viruses
- Species Specific Viruses
  - HIV type 1 & 2
  - Human T-lymphotropic virus 1 & 2
  - Human cytomegalovirus
  - Human herpes virus
  - Human hepatitis B & C
  - Simian virus 40
  - Adeno associated virus
  - Epstein-Barr virus

- Bovine/porcine viruses
  - Bovine diarrhea virus
  - Infectious bovine rhinotrachitis virus
  - Para-influenza virus
  - Porcine parvovirus

- Retrovirus assays
  - Reverse transcriptase
  - Transmission electron microscopy
  - S+L- and XC plaque

- Prions
  - PrPcSC negative
  - No mutations in PrP
  - 129 V/M (heterozygous)

NO CONTAMINANTS FOUND

PER.C6® Cell Line Generation Process

1. GOI Sequence Optimization & Vector Preparation
2. Transfection
3. Stable Pool & Material Generation
4. LDC, Clone Screening & Expansion
5. Create RCB
6. Stability Study
7. Process Development & Implementation
8. FB Clone Screening & Selection

Create MCB
Cell Culture Tool Box

Objective: Establish a tool box of platform processes from which the optimal process may be chosen for each program

Right tool for every job!
- Selection of process based on process requirements as dictated by product, clone characteristics, etc.
- Familiar and easy to implement

PER.C6® Platform Production Processes

Available tools
- Batch - quick and easy
- Fed-batch - standard production work horse
- XD® - constant environment and extreme productivities

Safety and Consistency
- Free of animal-derived material or hazardous chemicals
- Chemically defined throughout
PER.C6® Fed-Batch Historical View

Commercial batch medium ($X_{v_{\text{max}}} \sim 5$-$6$ million cells/mL, titer $\sim 400$ mg/L)

Combination of commercial feed and in-house designed cocktails
- Include hydrolysates, amino acids, trace element cocktails, etc.
- Multiple bolus shots applied throughout the process
- $X_{v_{\text{max}}} \sim 15$ million cells/mL
- Typical titer $\sim 1.5$ – 2 g/L in fed batch

Platform Fed-Batch Attributes

“Generic”, chemically-defined batch media intended for multiple cell lines with feed media a concentrated form of batch media components

Simple feeding strategy for multiple cell lines and scales

Small scale model which is predictive of fed-batch bioreactor performance
Platform Batch and Fed-Batch Development
End Result

![Graph showing Xv (E6 viable cells/mL) and Product Titer (g/L) over time (Days)].

**Small-Scale Fed-Batch System**

Easy to set up and manipulate - “high” throughput

- Small volume (50 mL in 250 mL Shake Flask)
- Minimal manual intervention/process analytical requirements (Feeding/sampling once daily or every other day)

*Predictive* of fed-batch bioreactor performance

**Applications**

- Clone screening
- Media/process development
- Process troubleshooting
The XD® Process

Extreme Density Cell Culture

Fresh Medium

Cells and Product

ATF System

Spent Medium

Image from www.magellaninstruments.com

XD® Process Development

Cell Concentration (E6 cells/mL)

Time (days)

- Medium A
- Medium B
- Medium C
From 250 mL Shake Flask to 250 L Bioreactor

EXPERIENCES WITH THE PER.C6® PLATFORM
FED-BATCH PROCESS
Three Processes:
Protein A Capture Process
CEX Capture Process
Disposable Process

CASE STUDY: COMPARISON OF TRADITIONAL AND NON-TRADITIONAL DOWNSTREAM PROCESSES

Process Overview – Base Case (Protein A Capture)
Process Overview – CEX Capture

- Sedimentation
- Depth Filtration
- Cells
- Cell debris
- HCP Concentration Buffer exchange
- HCP DNA
- DNA Virus
- HCP DNA
- VI
- HIC
- FT
- MF
- TFF
- PPT
- SartobindPhenyl
- HIC FT
- Natrix C
- Bind/Elute
- Cells
- Cell debris
- HCP Concentration Buffer exchange
- HCP DNA
- DNA Virus
- HCP DNA
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- Bind/Elute
- HCP DNA
- DNA Virus
- Aggregate
- Cells
- Cell debris
- HCP Concentration Buffer exchange
- HCP DNA
- DNA Virus
- Aggregate
- HCP DNA
- DNA Virus
- Aggregate
Process Performance – Recovery and Aggregate Removal

Scalability of Disposable Process

Note: Starting material was from a different reactor than previous case study
Summary
PER.C6® Platform Technology

Industrially-Relevant Cell Line Generation Timeline and Yields
Small-Scale Fed-Batch System Predictive of Bioreactor Performance

Scalable and Disposable Downstream Processes
High-yielding, Reproducible Production Processes

PER.C6® Based Products in Clinical Trials

<table>
<thead>
<tr>
<th>Sponsors</th>
<th>Products</th>
<th># Studies</th>
<th># Subjects (incl. placebo)</th>
<th>Countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>AME/El Lilly, AERAS, Ark Therapeutics, Berlex/Schering, FLUPAN, Genvec, JAKS, Merck&amp;Co, M. Laboratories, Neotropix, NIH-VRC, NIH-AID, NIH-IPCAVD, Sanofi pasteur, Selective Genetics, Transgene</td>
<td>Gene Therapy Vaccines mAbs Recombinant Proteins</td>
<td>~40*</td>
<td>&gt;5,500</td>
<td>US, Canada, Belgium, Finland, France, Germany, Hungary, Kenya, Norway, Netherlands, Sweden, Switzerland, UK, Malawi, South Africa, India, Thailand, Australia, Brazil, Peru, Philippines, Puerto Rico, Dom. Rep, Haiti, Jamaica</td>
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List of Abbreviations

Ad5 Adenovirus serotype 5
HEK Human Embryonic Kidney
CHO Chinese Hamster Ovary
GOI Gene of Interest
LDC Limited Dilution Cloning
FB Fed-Batch
RCB Research Cell Bank
MCB Master Cell Bank
Xv Viable Cell Concentration
ATF Alternating Tangential Flow
HF Hollow Fiber
IgG Immunoglobulin G
SF Shake Flask
BRX Bioreactor
HCP Host Cell Protein
PrA Protein A
PPT PEG Precipitation
MF TFF Microfiltration Tangential Flow Filtration
CEX Cation Exchange
AEX Anion Exchange
FT Flow Through
VI Viral Inactivation
HIC Hydrophobic Interaction Chromatography
AE Adverse Effects