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Vaccine Technology IV, Session III: Late stage and recently launched vaccines

Process Understanding Approach for a Late-Stage Recombinant Protein Vaccine Produced in *Saccharomyces cerevisiae*

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Nedim Altaras[1], Brian E. Mickus[2], José Manuel Otero[1,3]

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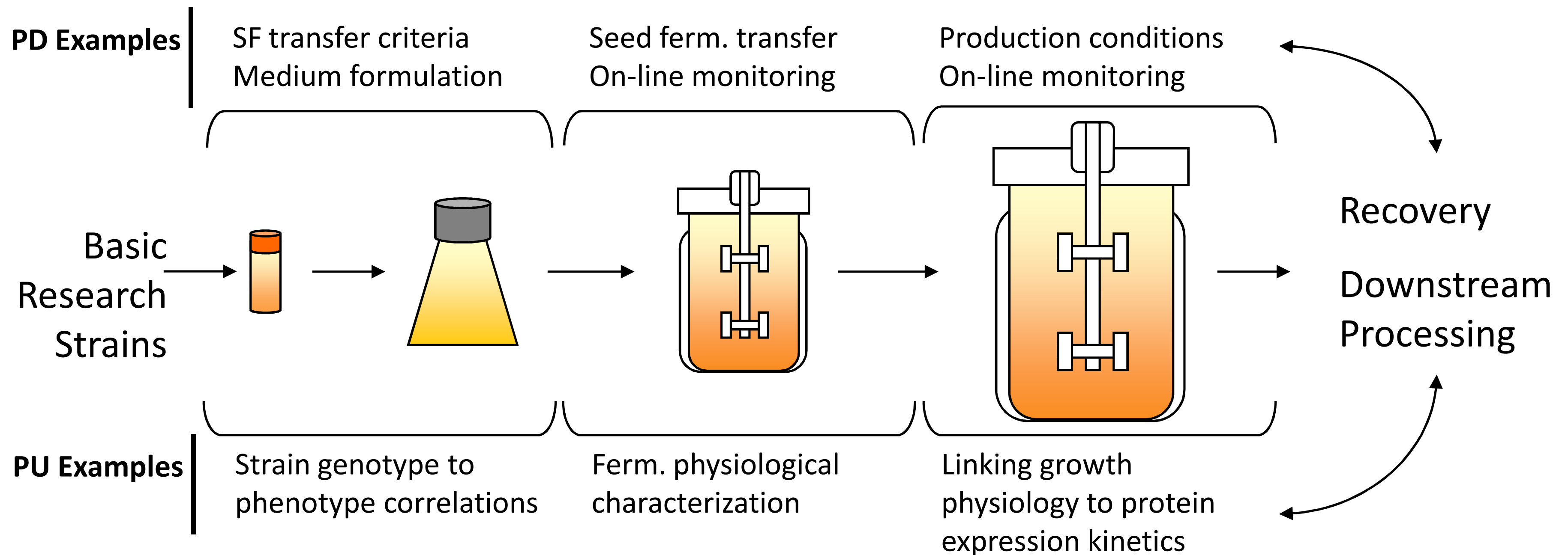
MERCK & CO., INC.
Whitehouse Station, N.J., U.S.A.

Process Development vs. Process Understanding

Process Development (PD) Objective:

– **Design robust, controllable, and scalable processes to optimize productivity and deliver clinically driven product quality attributes.**



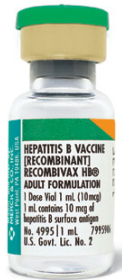
Process Understanding (PU) Objective:

– **Identify critical inputs & outputs, and attempt to elucidate mechanistic understanding to improve existing manufacturing processes or *de novo* process development.**

MERCK Vaccine R&D

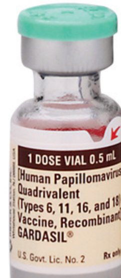
S. cerevisiae: A Merck Vaccine Manufacturing Platform

Since 1980s to Present



Recombivax HB®
[Hepatitis B Vaccine [Recombinant]]

•1st vaccine to lead to link viral infection and carcinoma



GARDASIL
[Human Papillomavirus Quadrivalent (Types 6, 11, 16, and 18) Vaccine, Recombinant]

•Virus-like particle (VLP) to prevent HPV infections, pre-cancerous lesions,

Investigational *S. aureus* Vaccine (SAV)

Physiological differences across all three programs were observed

- **SAV:** Significantly reduced galactose metabolism during induction

•1986: FDA appr

•Non-infectious vaccine derived surface antigen in *S. cerevisiae.*

•Unmet medical

- •US 4-5000 deaths ann.
- •Globally >1 million deaths ann.

- **Fed-batch fermentation process with complex medium (YE, soy peptone, dextrose, amino acids)**

•Unmet medical need:

- •US 270,000 women died ann. (2002)
- •Globally >0.5 million deaths ann.

•Leveraged RecombivaxHB process; chemically-defined fermentation medium

iron
xpressed in

andidate

•3 L, 15 L process development through process validation at manufacturing scale

•Leveraged RecombivaxHB and GARDASIL genetic engineering and process development

Process Understanding in an Industrial Context

- **Genome sequencing could reveal opportunity for genetic enhancement, but resource cost to implement would be high**:
  - Reproduce cGMP cell banks
  - Potential for additional clinical studies

- **Process understanding opportunity**:
  - Can we employ physiological characterization to better understand **process robustness?**
  - Can a process modification (e.g. medium, operating parameter) be employed to compensate for undesirable phenotype?

- **Process understanding to be completed with line of sight to long-term understanding and manufacturing support – a change in paradigm**:
  - Process development does not end with transfer to manufacturing
  - Life-cycle management requires that processes evolve with improved technologies, analytics, and increased demand

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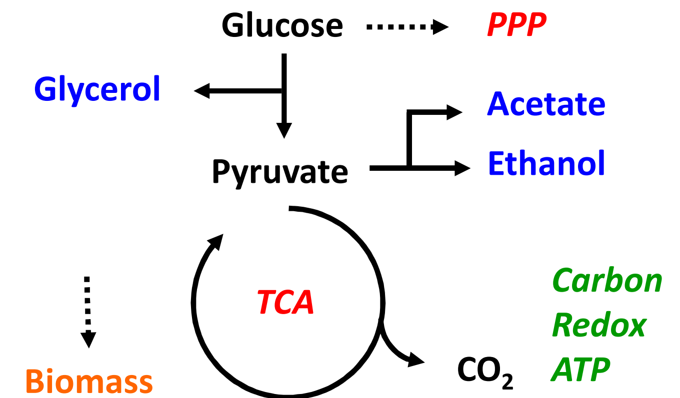
S. cerevisiae Basics: Central Carbon Metabolism

Complex glucose signaling regulatory network:

- **Crabtree effect (aerobic):**
  - **Respiration:**
    - Low extracellular glucose
    - Glycolytic flux with no pyruvate accumulation
    - TCA cycle flux is high
  - **Fermentation:**
    - High extracellular glucose
    - Glycolytic flux with high pyruvate accumulation
    - TCA cycle incapable of sustaining flux
    - Overflow to ethanol, acetate, and glycerol
- **Glucose genetic regulation:**
  - **Derepression:**
    - Low extracellular glucose
    - Signaling cascade to up-regulate large/diverse gene clusters (e.g., TCA cycle)
    - Required for galactose metabolism and induction of protein expression.
  - **Repression:**
    - High extracellular glucose
    - Signaling cascade to down-regulated large/diverse gene clusters



High Glucose
Fermentative
RQ > 1

Y_{SX} = 0.17
Y_{SEtOH} = 0.47
Y_{SAcet} = 0.01
Y_{SGlyc} = 0.07
Y_{SCO2} = 0.23

C-balance: ≥ 95%
μ_{max} = 0.33 h^{-1}

Low Glucose
Respiration
RQ ≤ 1

Y_{SX} = 0.51
No ethanol, acetate, glycerol production

μ_{max} < 0.33h^{-1}

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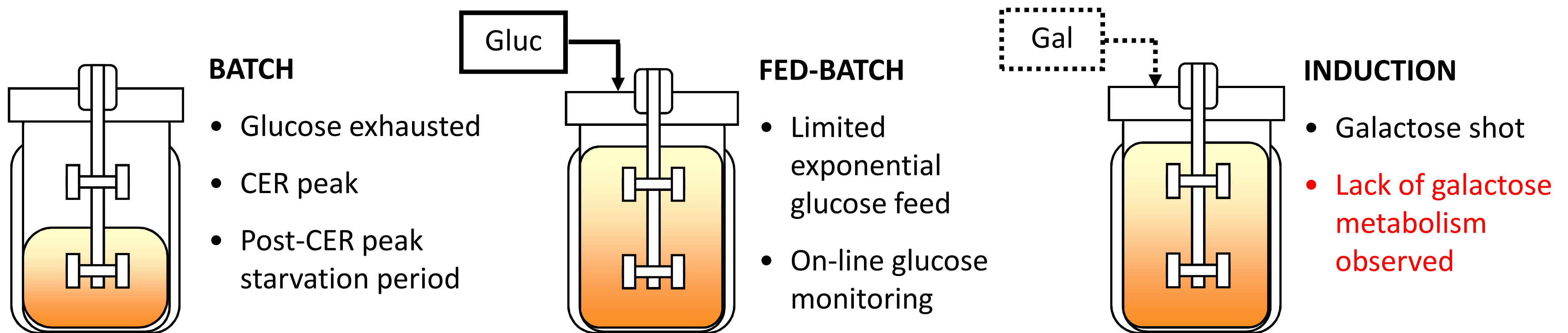
S. cerevisiae CEN.PK113-7D data shown.
Otero, et al, 2009 PhD thesis.

SAV Upstream Process Overview



BATCH

- Glucose exhausted
- CER peak
- Post-CER peak starvation period

FED-BATCH

- Limited exponential glucose feed
- On-line glucose monitoring

INDUCTION

- Galactose shot
- Lack of galactose metabolism observed

CER OUR Rq

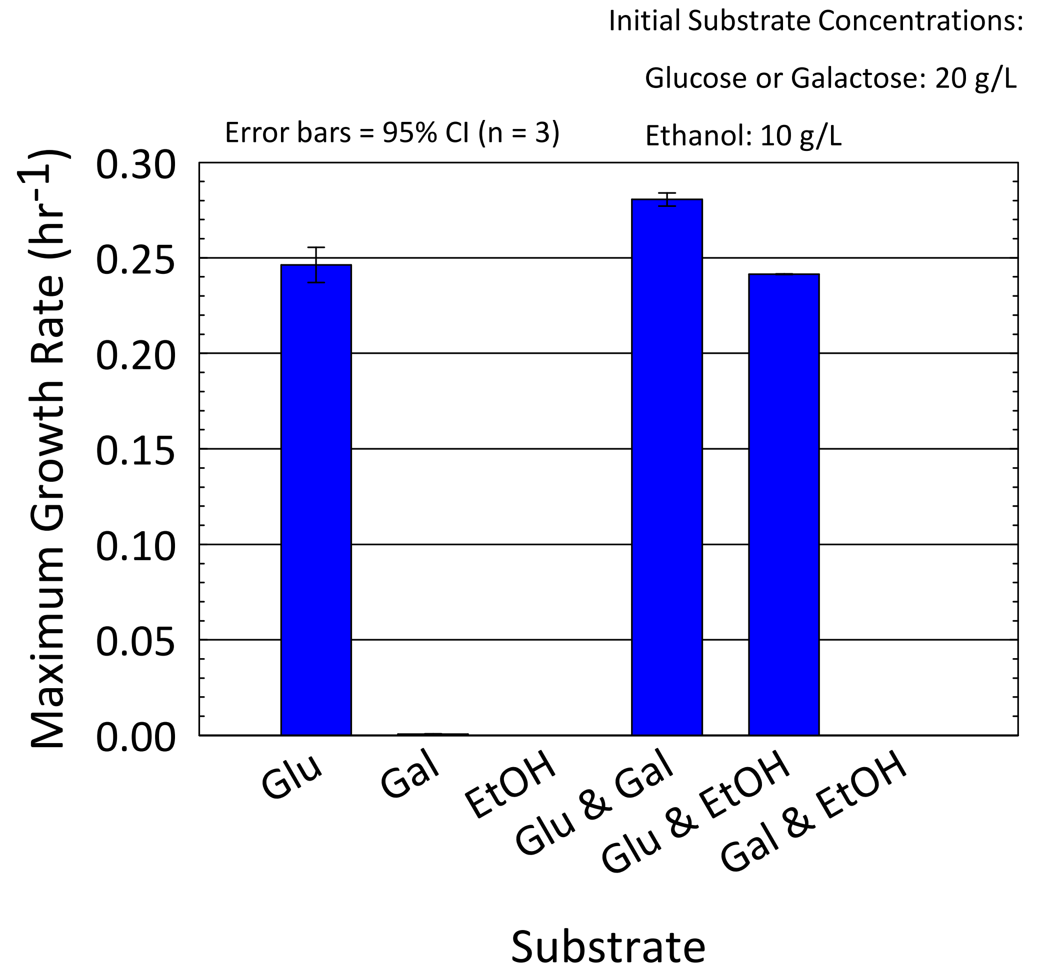
CER/OUR (mmol/L/hr)

Rq

Batch Age (hrs)

Process Understanding Shake Flask Experiments: Growth Evaluation on Various Carbon Sources

- The lack of growth on ethanol in addition to galactose indicates that the expression strain has a respiratory deficiency
- Protein expression is not detrimental to growth in comparison to the NORF strain (data not shown)
- No protein production without galactose – no leaky expression
- Galactose uptake must be occurring in order to promote transcription of the antigen gene (data not shown)



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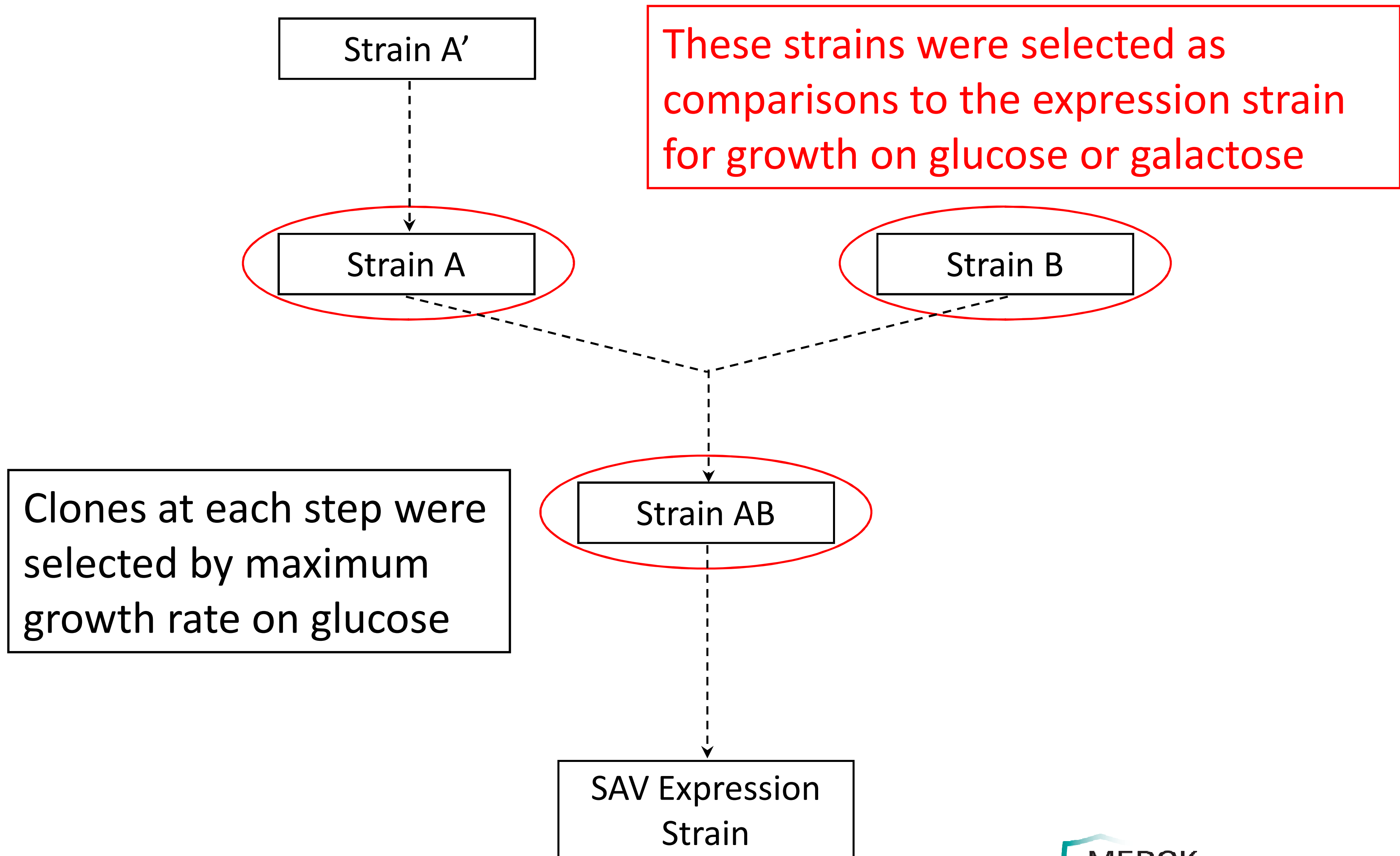
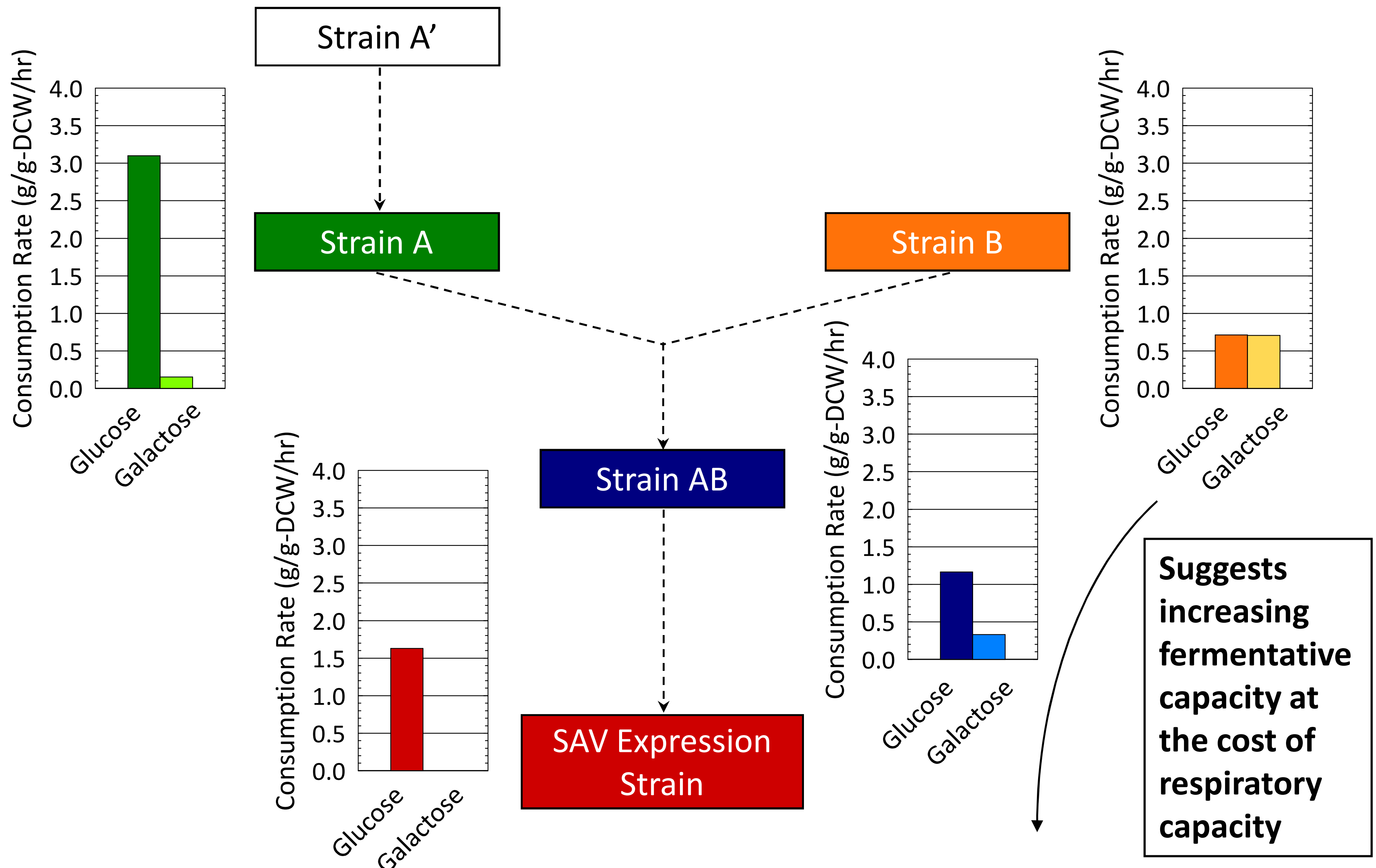
Expression Strain Lineage



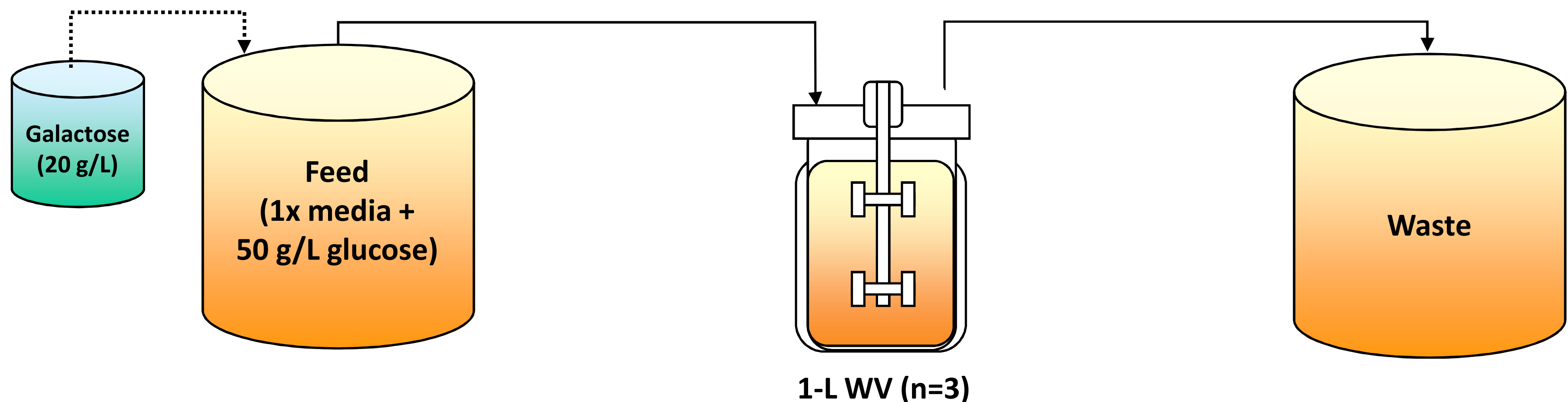
Note: Strain B is the parent for RECOMBIVAX HB® and GARDASIL™

Respiratory Capacity of Parental Strains



Chemostat: A Process Understanding Tool

- Continuous addition of medium and removal of culture allows the growth rate to be controlled and maintained at steady state
- Dilution rate = 0.2 h^{-1}
  - μ_{max} of strain was determined to be 0.29 h^{-1} during batch culture
- Can determine the impact of galactose addition in the presence of a consumable carbon source (glucose)
- Transcriptome and exo-metabolome samples taken during glucose steady state and galactose steady state allow us to look for differences in gene expression and metabolites



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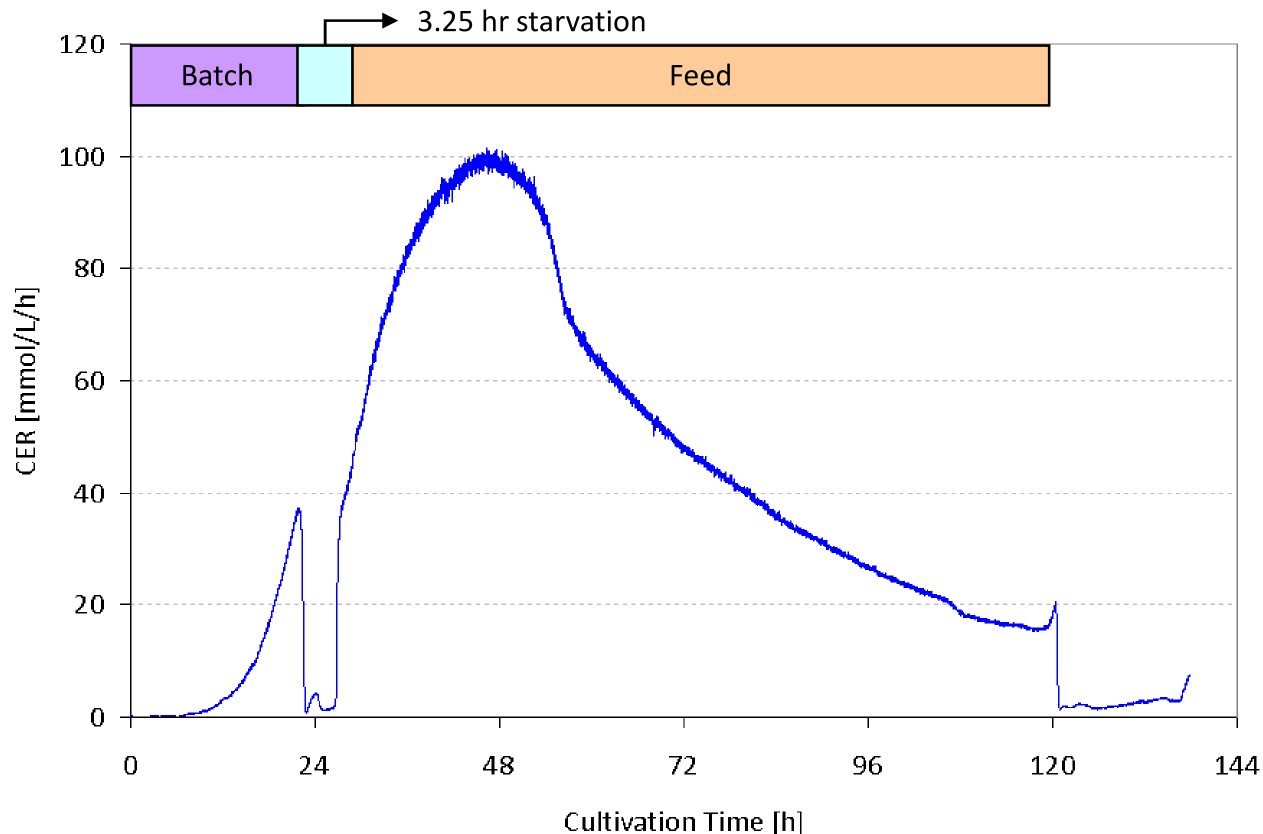
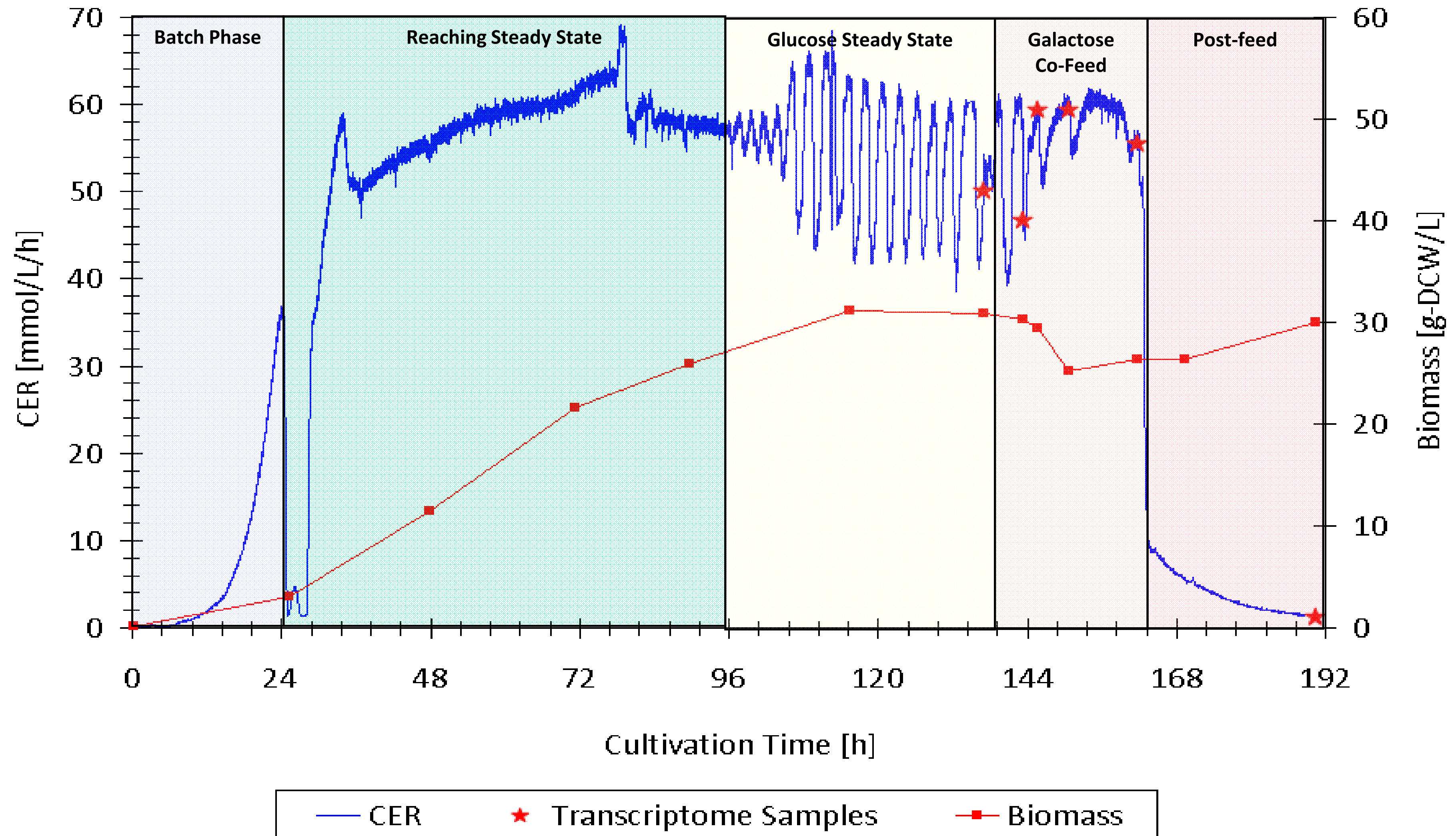
Chemostat CER Profiles (D = 0.2 h^{-1})

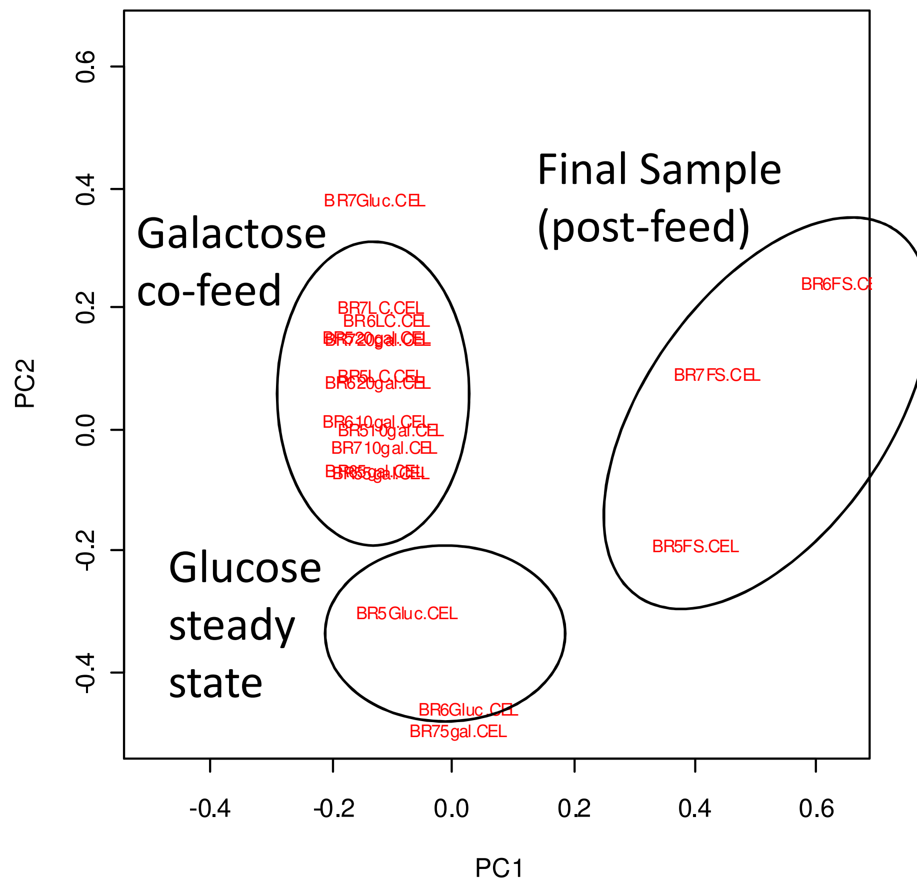


- Cell-specific growth rate was slower than glucose-limited feed rate resulting in washout
- Confirms respiratory deficiency of the strain, since the **fermentative** growth rate, which was demonstrated to be significantly higher than 0.2 h^{-1}, could not be maintained in a respiratory regime
- Glucose feed rate during the fed-batch process is equivalent to a cell-specific growth rate of 0.13 h^{-1} (cells are able to respire, RQ = 1)
- **Try repeating the chemostat experiment with D = 0.1 h^{-1}!**

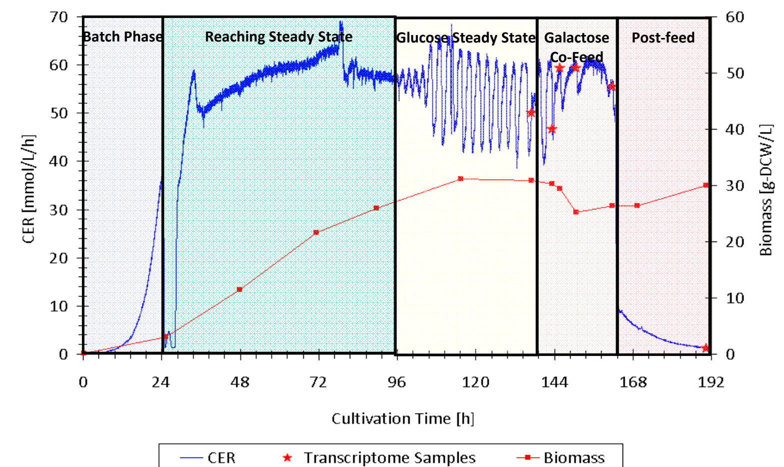
Chemostat CER and Biomass Profiles (D = 0.1 h^{-1})



Principle Component Analysis (PCA)



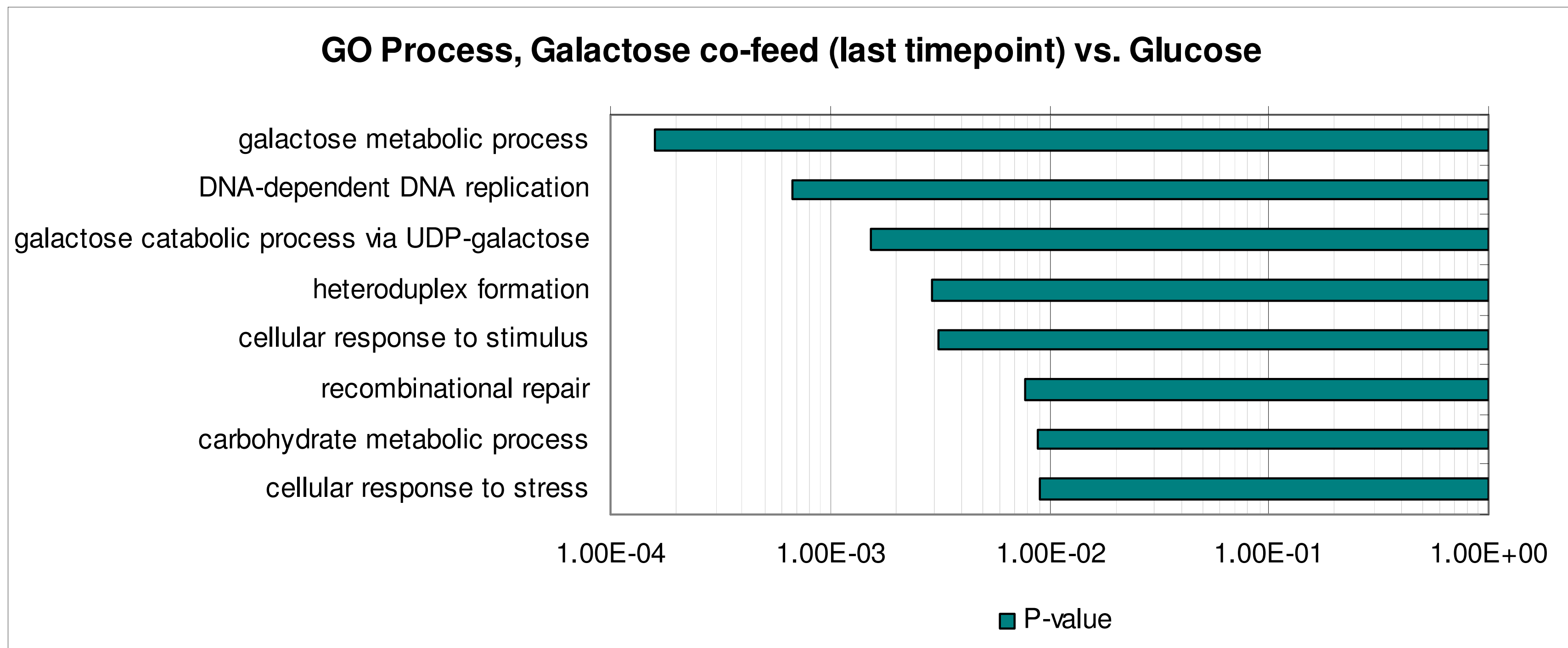
- Affymetrix Yeast 2.0
- Transcriptome samples grouped based on different phases of the process:
  - Glucose steady state
  - Galactose co-feed
  - Final sample (post-feed)



Gene Ontology:

Galactose Co-Feed vs. Glucose Steady State

- Contrary to previous hypothesis, the presence of galactose does not cause any abnormal gene expression response by the strain
- Response was typical for a galactose-consuming strain



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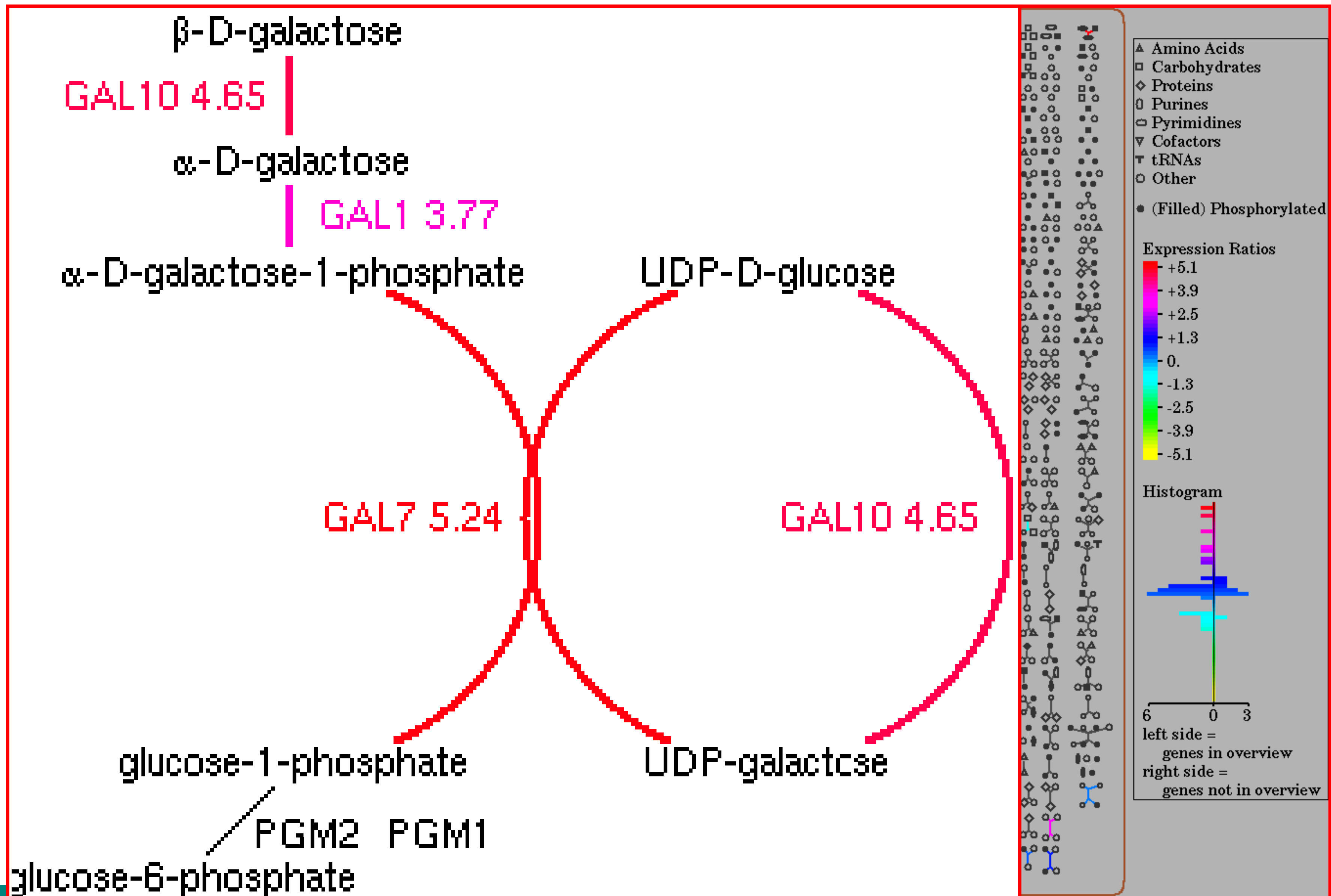
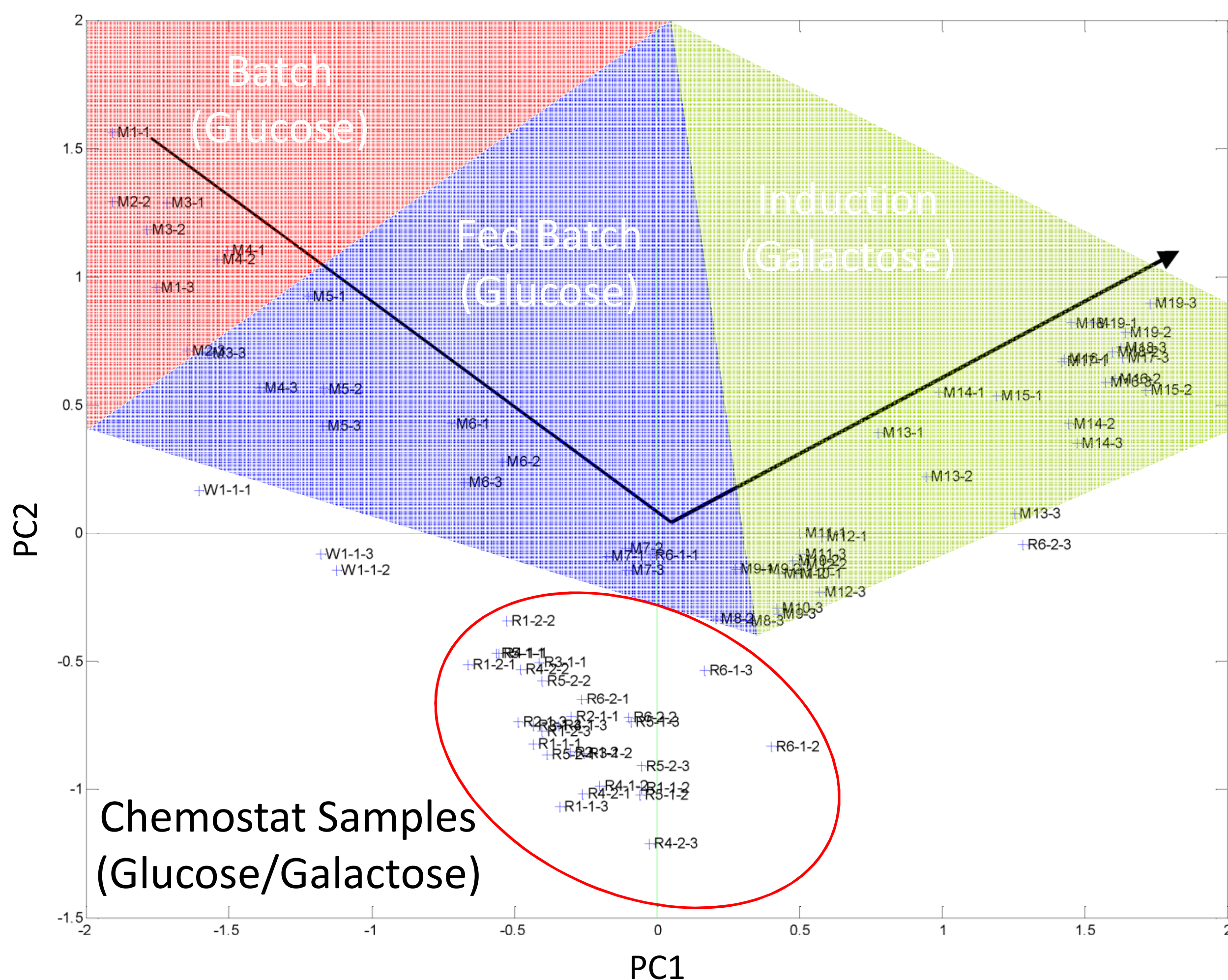
Metabolic Pathways:
Galactose Co-feed vs. Glucose Steady State



Principle Component Analysis of Exo-Metabolome



Quantification of 63 metabolites and elements for chemostat experiment samples and manufacturing samples (3 lots at mfg. scale)

There is a clear grouping by process step in the manufacturing samples with respect to the principal components.

This grouping could not be reproduced in the chemostat, even by perturbing the system with galactose addition.

Exo-Metabolome analysis courtesy of TNO Innovation for Life

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Conclusions

- Confirmed respiratory deficiency of the expression strain
- Respiratory capacity was reduced during expression strain development
- Transcriptome analysis revealed no dysfunction in transcription of any of the major galactose metabolic pathway genes
- Exo-metabolome analysis demonstrated little variation in any of the chemostat samples when compared to the manufacturing process

Impact: Why should process development invest in understanding?

- **Defining experimental space:**
  - Where **not** to invest resources is just as valuable as determining where to invest resources.
  - **Hypothesis:** Altered physiology observed was due to addition of galactose and subsequent metabolism
  - **Conclusion:** False. More significant strain differences observed.

- **Regulatory agency expectations are increasing:**
  - Life-cycle management requires that we continuously invest in our franchises. It's not about what the FDA requires today, but what questions will it ask 10-20 years from now?

- **Invest in expression systems engineering:**
  - Process development groups may have 'preferred' cell substrates from historical programs, but assumptions should be challenged with newly available tools (deep sequencing, bioinformatics, phenotype/genotype relationships).
  - **Example:** RecombivaxHB (1986), Gardasil (2006), Investigational SAV (2011)

Acknowledgements

- **Merck & Co., Inc.**
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  - Machtelt Braaksma
  - Leon Coulier
  - Karin M. Overkamp
  - Bianca van der Werf-van der Vat

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Back-up Slides

Recombivax HB®

- Non-infectious subunit viral vaccine derived from hepatitis B surface antigen.
  - Viral gene encoding HBsAg (*adw* serotype) cloned into *S. cerevisiae*
  - Complex fermentation medium (yeast extract, soy peptone, glucose, amino acids, salts).
  - Non-secreted product → requires cell disruption

- *S. cerevisiae* Advantages
  - Easy regulatory approval due to *GRAS* status
  - Laboratory process development easily scaled up to >1000L fermentations
  - Process developed during 1980s
    - *Successful even without today's advances → robust!*

All information presented is publicly available:
www.merck.com/product/usa/pi_circulars/r/recombivax_hb/recombivax_pi.pdf

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Gardasil™

- Human Papillomavirus (HPV)
  - Many viral types leading to diverse infection states
    - HPV Types 6/11 → genital warts
    - HPV Types 16/18 (31/45/52/58/33) → cervical cancer
- Non-infectious major surface protein (L1 protein, 55-57 kDa) of HPV viral capsid encoded in *S. cerevisiae*
  - Intracellular expression in *S. cerevisiae*
  - L1 protein of HPV Types 6/11/16/18 → independent fermentations lead to formation of **virus-like particle (VLP)**
  - Different VLPs then mixed to form Gardasil™
  - Clinical efficacy thus far – 99.99%
- Gardasil™ Quadrivalent approved in US and EU
  - Recommended by USA FDA to be reviewed as a mandatory vaccine
  - Analysts expect sales >$ 1 billion USD
- Development on 2nd generation HPV vaccines and process actively underway

All information presented is publicly available: www.gardasil.com

S. cerevisiae Basics: Central Carbon Metabolism

Complex glucose signaling regulatory network:

- **Crabtree effect (aerobic):**
  - **Respiration:**
    - Low extracellular glucose
    - Glycolytic flux with no pyruvate accumulation
    - TCA cycle flux is high
  - **Fermentation:**
    - High extracellular glucose
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    - TCA cycle incapable of sustaining flux
    - Overflow to ethanol, acetate, and glycerol
- **Glucose genetic regulation:**
  - **Derepression:**
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  - **Repression:**
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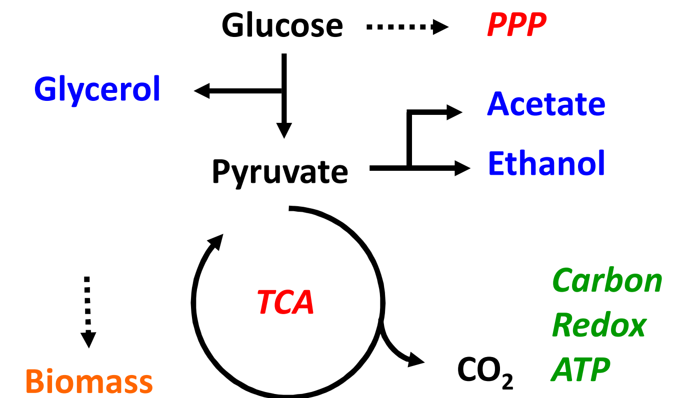

High Glucose	**Low Glucose**
Fermentative	**Respiration**
RQ > 1	**RQ ≤ 1**
$Y_{SX} = 0.17$	$Y_{SX} = 0.51$
$Y_{SEtOH} = 0.47$	**No ethanol, acetate, glycerol production**
$Y_{SAcet} = 0.01$	
$Y_{SGlyc} = 0.07$	
$Y_{SCO2} = 0.23$	
C-balance: ≥ 95%	
$\mu_{max} = 0.33\ h^{-1}$	$\mu_{max} < 0.33 h^{-1}$

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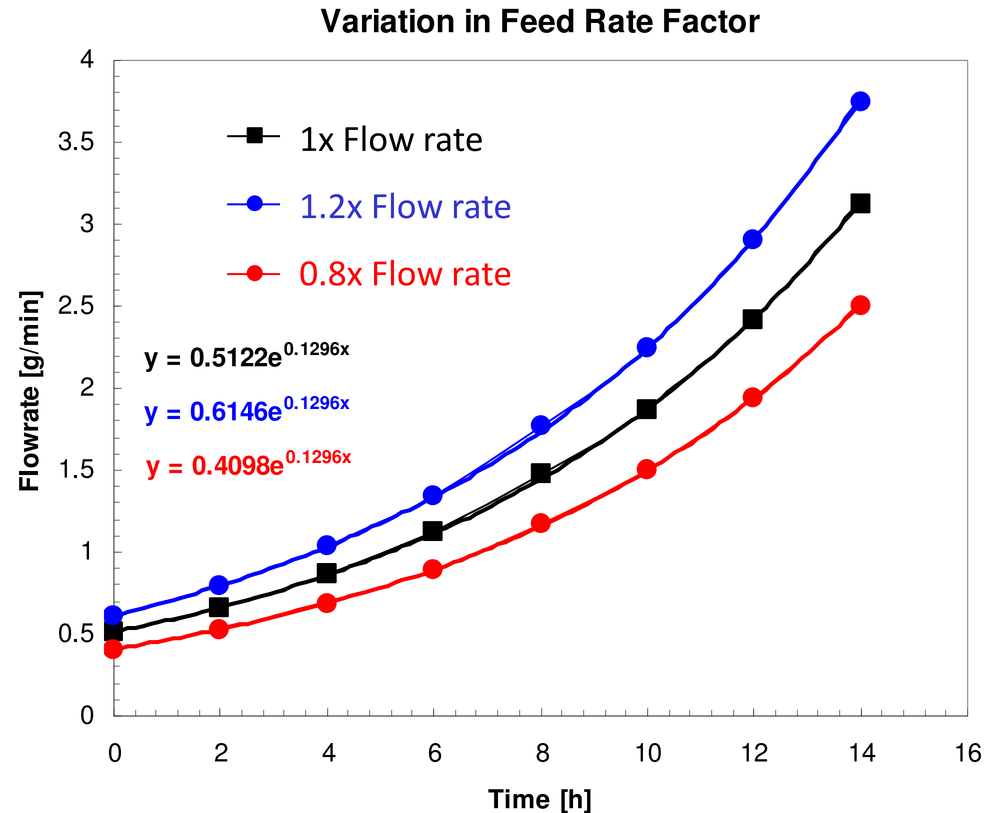
S. cerevisiae CEN.PK113-7D data shown.
Otero, et al. 2009 PhD thesis.

Critical Control Parameter: Glucose Feed Rate

- Glucose Feed Rate Factor:
  - GARDASIL™ Process Development: The specific growth rate was determined based on oxygen uptake rate. This process used same feed rate profile – no consideration for strain difference.
  - Process Understanding: Determined specific growth rate based on direct measurement of biomass – OD_{600} and dry cell weight.



Changes in the feed rate factor (0.8x, 1.2x) do <u>not</u> change the <u>exponential rate</u> of glucose feed → it is the <u>exponential rate of feed</u> that correlates to physiological growth rate

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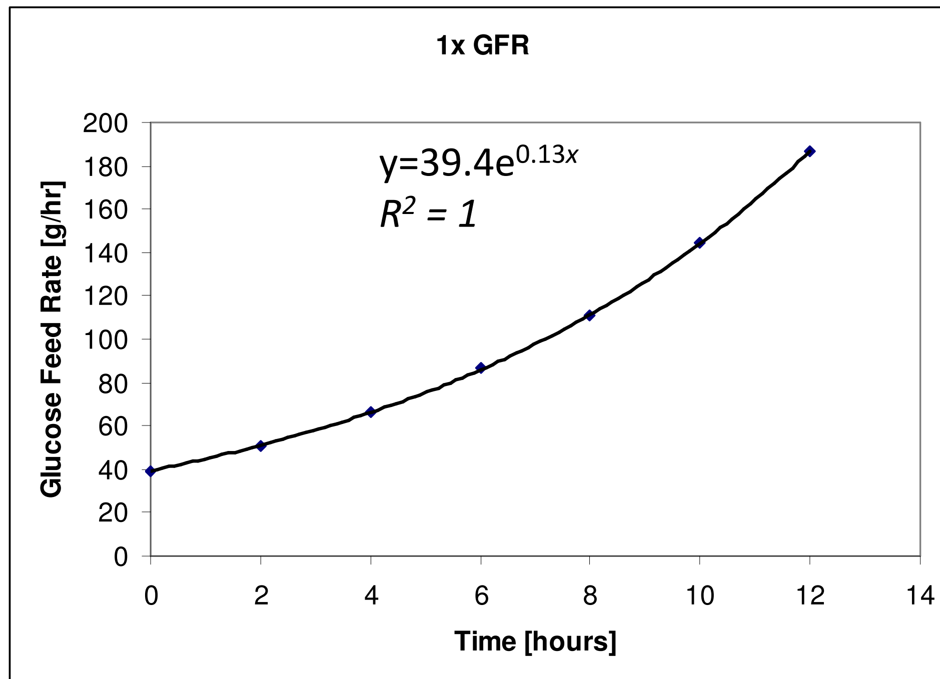
So why does the manufacturing process work?

- The manufacturing process utilizes a glucose limited exponential feed
  – The feed rate profile was determined based on GARDASIL™ Process Development with no consideration for strain difference
  – Glucose feed rate is equivalent to a cell specific growth rate of 0.13 h^{-1}
  – Cells are able to respire during fed-batch (RQ = 1)



Explains why respiratory deficiency observed could not be reconciled with fed-batch profile

Try repeating the chemostat experiment with D = 0.1 h^{-1}!

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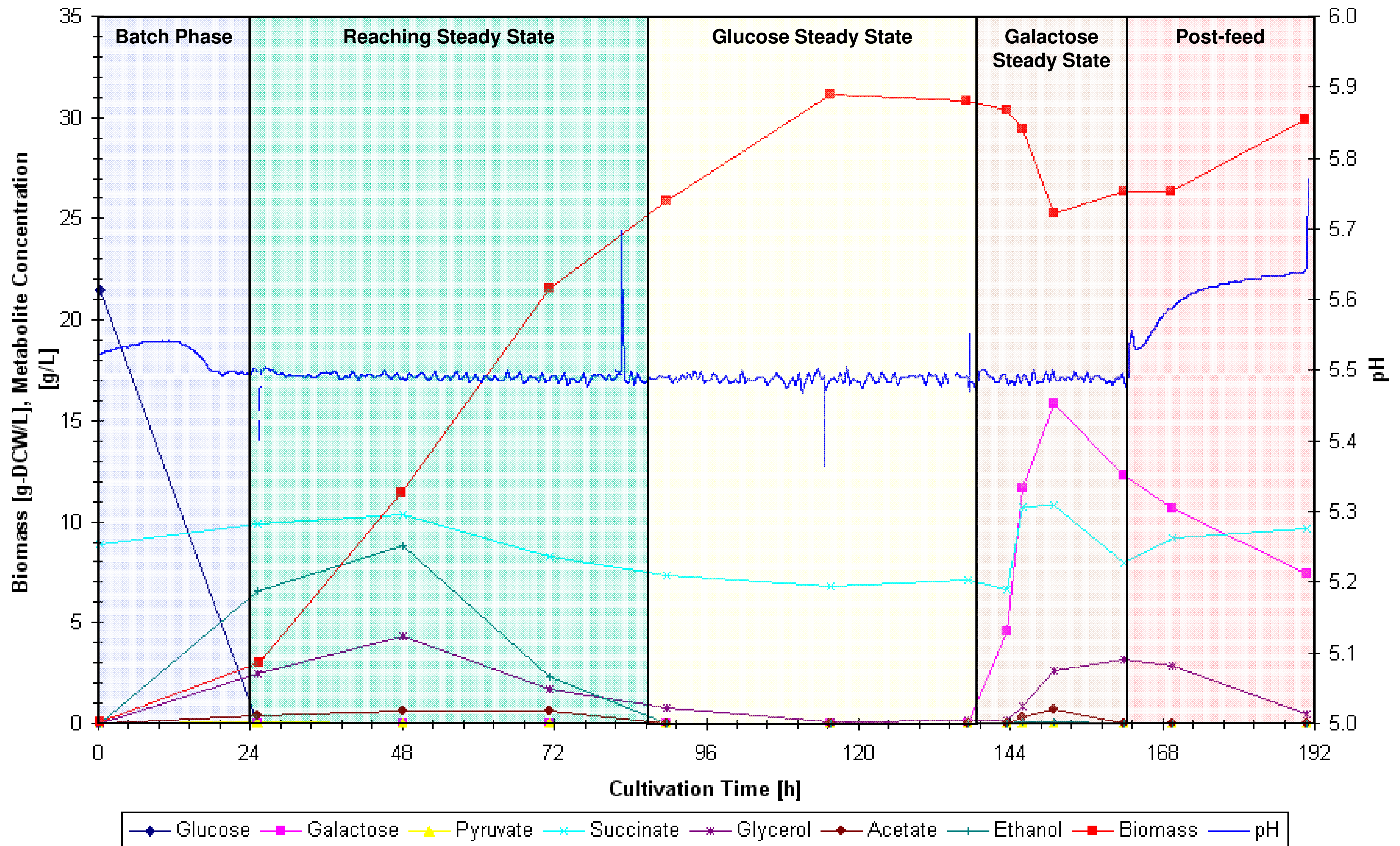
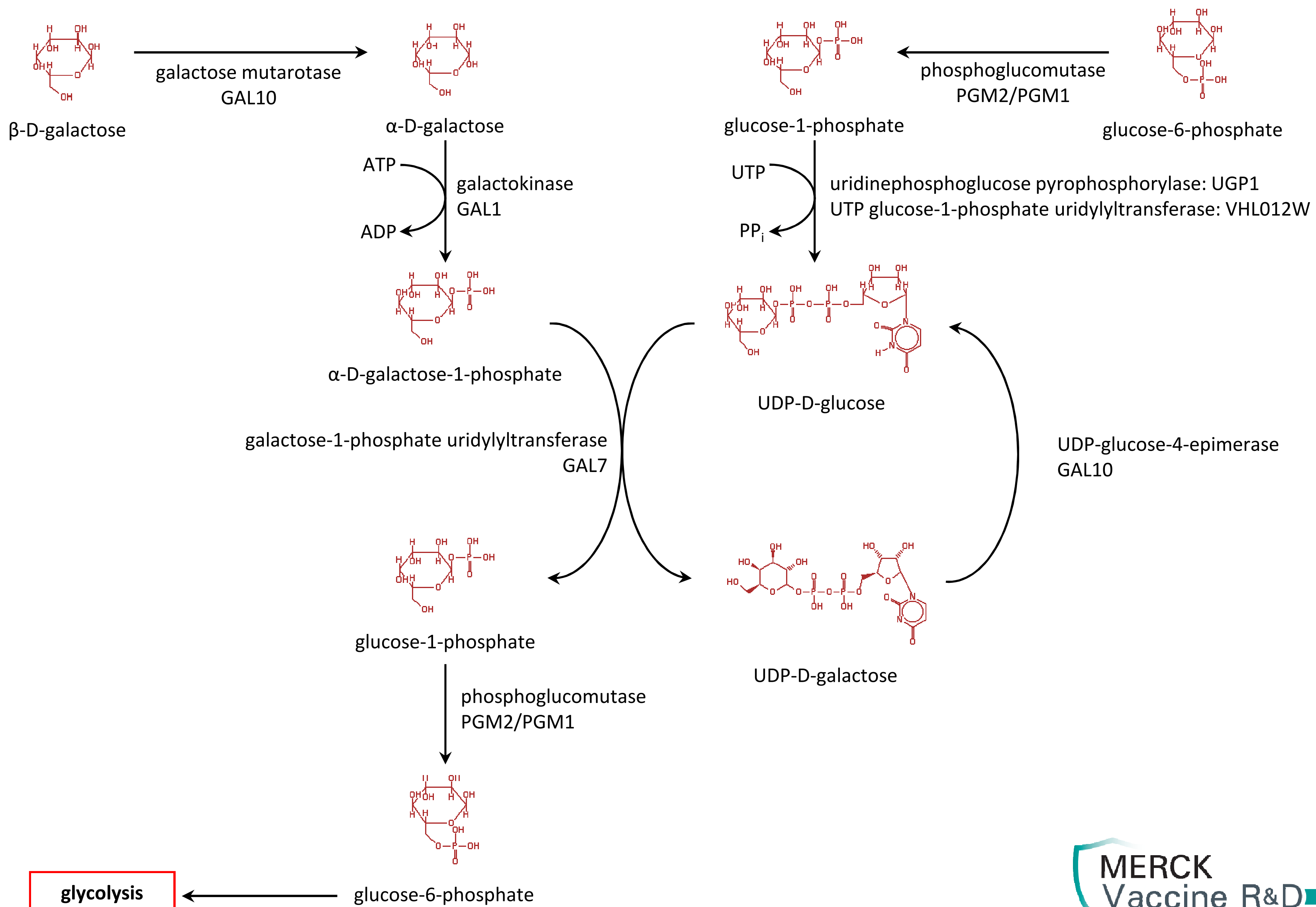

BR7 Metabolites
Batch Phase
Reaching Steady State
Glucose Steady State
Galactose Steady State
Post-feed
Biomass [g-DCW/L], Metabolite Concentration [g/L]
pH
Cultivation Time [h]
0
5
10
15
20
25
30
35
24
48
72
96
120
144
168
192
5.0
5.1
5.2
5.3
5.4
5.5
5.6
5.7
5.8
5.9
6.0
Glucose
Galactose
Pyruvate
Succinate
Glycerol
Acetate
Ethanol
Biomass
pH

Galactose Metabolic Pathway



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S. cerevisiae Transcriptional Profiling

- Well-annotated genome
  - First eukaryotic genome fully sequenced (Goffeau, et al. Science. 1996)
  - *Saccharomyces* Genome Database (www.yeastgenome.org)
    - Collects information and maintains a database of the molecular biology of *S. cerevisiae*
- Genome-scale metabolic model published (Förster, et al. Genome Research. 2003)
- Affymetrix platform
  - Yeast Genome 2.0 Array – contains probe sets to detect transcripts from *S. cerevisiae* and *S. pombe*
  - Used in most major yeast laboratories
- Molecular Profiling
  - Merck acquired Rosetta in 2001
  - Leaders in DNA array technology

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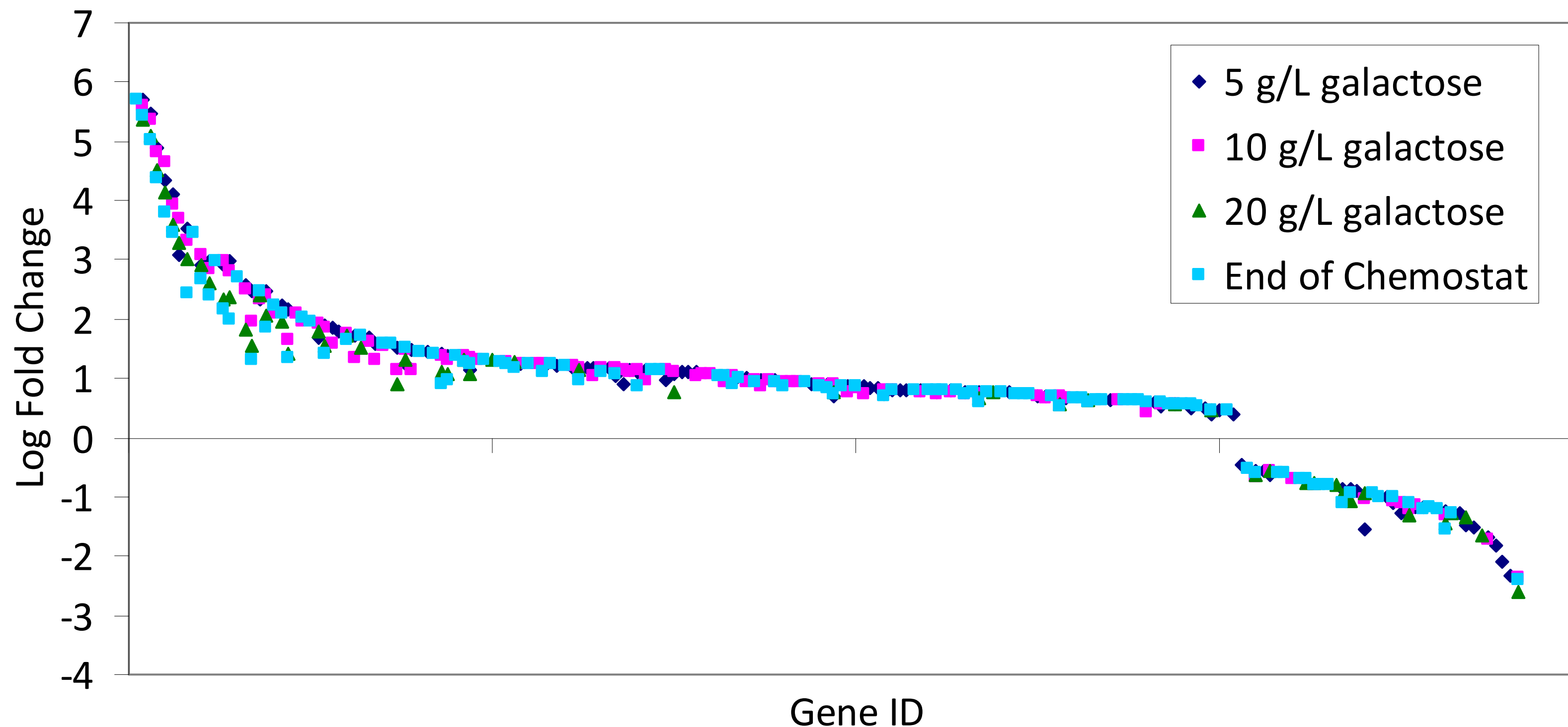
Transcriptional Comparison of Galactose Conditions to Glucose Steady State



- There does not appear to be a "dose-response" effect in terms of transcriptional levels as galactose concentration increases
- All galactose co-feed conditions can be combined into one galactose group
  - Increases statistical power of comparison to glucose

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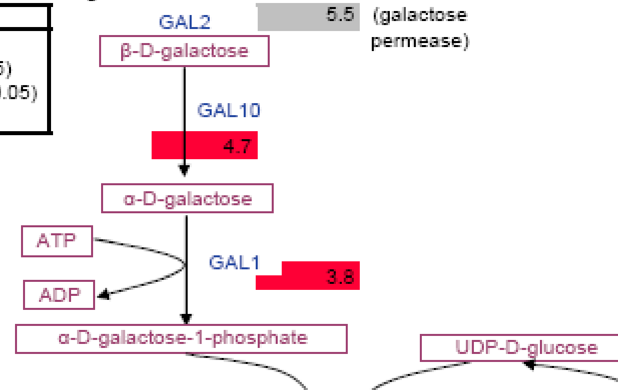
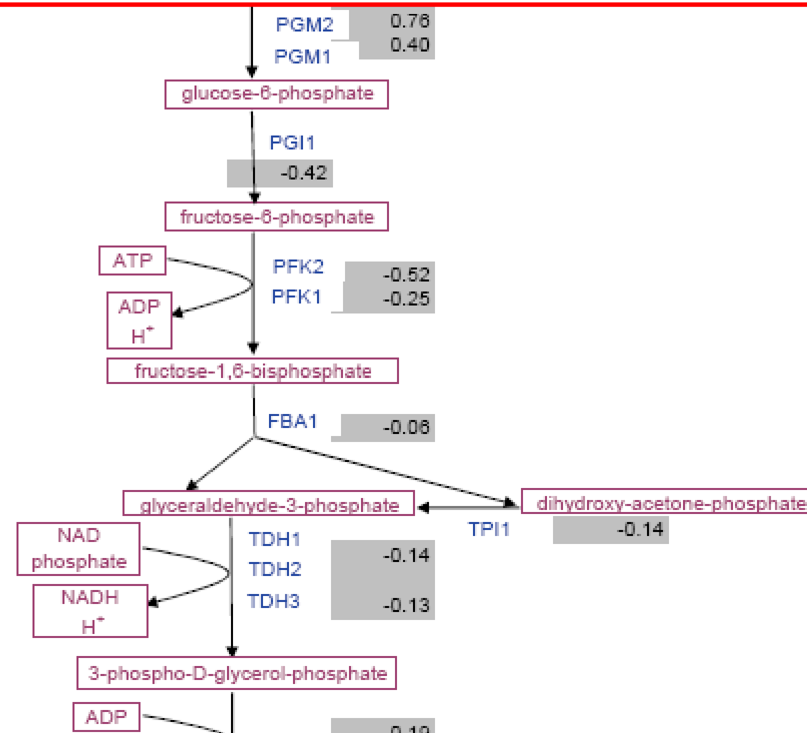
Data in boxes represent Log FC of galactose co-feed vs. glucose

Key:	
	Log FC not significant (p>0.05)
	Log FC significantly upregulated (p≤0.05)
	Log FC significantly downregulated (p≤0.05)
Blank	Gene not included in top genes list

Regulatory Genes	
GAL4	
GAL80	1.2
GAL3	1.1
GAL6	0.72
MIG1	0.56



Adding galactose to the medium when there is a consumable carbon source present produces the expected transcriptional response. Galactose does not appear to impact the rest of central carbon metabolism at a transcriptional level.



R&D

Metabolites Quantified in Exo-Metabolome Analysis

Metabolite	Method
Alanine	AminoTac
Arginine	AminoTac
Asparagine	AminoTac
Aspartic Acid	AminoTac
Cysteine (1)	AminoTac
Glutamic Acid	AminoTac
Glutamine	AminoTac
Glycine	AminoTac
Histidine	AminoTac
Isoleucine	AminoTac
Leucine	AminoTac
Lysine	AminoTac
Methionine	AminoTac
Phenylalanine	AminoTac
Proline	AminoTac
Serine	AminoTac
Threonine	AminoTac
Tryptophan	AminoTac
Tyrosine	AminoTac
Valine	AminoTac

Metabolite	Method
Boron	ICP-AES / ICP-MS
Calcium	ICP-AES / ICP-MS
Cobalt	ICP-AES / ICP-MS
Copper	ICP-AES / ICP-MS
Iron	ICP-AES / ICP-MS
Magnesium	ICP-AES / ICP-MS
Manganese	ICP-AES / ICP-MS
Molybdenum	ICP-AES / ICP-MS
Nickel	ICP-AES / ICP-MS
Potassium	ICP-AES / ICP-MS
Sodium	ICP-AES / ICP-MS
Zinc	ICP-AES / ICP-MS
Adenine	OS-GC-MS
α-Ketoglutaric acid	OS-GC-MS
Citric acid	OS-GC-MS
Cytosine	OS-GC-MS
Fumaric acid	OS-GC-MS
Galactose	OS-GC-MS
Glucose	OS-GC-MS
Glycerol	OS-GC-MS

Metabolite	Method
Guanine	OS-GC-MS
Isocitric acid	OS-GC-MS
Lactic acid	OS-GC-MS
Linoleic acid	OS-GC-MS
Malic acid	OS-GC-MS
Myo-inositol	OS-GC-MS
Nicotinamide	OS-GC-MS
Nicotinic acid (Niacin)	OS-GC-MS
Oleic acid	OS-GC-MS
Oxalic acid	OS-GC-MS
Pantothenic acid	OS-GC-MS
Phosphate	OS-GC-MS
Phosphoenolpyruvate	OS-GC-MS
Pyridoxal	OS-GC-MS
Pyridoxamine	OS-GC-MS
Pyridoxine	OS-GC-MS
Pyruvic acid	OS-GC-MS
Succinic acid	OS-GC-MS
Thymine	OS-GC-MS
Trehalose	OS-GC-MS
Uracil	OS-GC-MS
NH_4^+ / NH_3	Colorimetric (2)

1. The AminoTac can only detect cystine, and not cysteine
2. Requires the colorimetric analysis of NH_4^+; based on the acid constant (pKa) of the NH_4^+/NH_3 couple the pH, the ratio between NH_4+/NH_3 can be calculated

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Biological Interpretation of Glycerol Yields

- In *S. cerevisiae*, glycerol is involved in
  - Balancing redox potential
  - Osmotic stress response
- Under **anaerobic conditions**, glycerol is produced to regenerate NAD^+ from excess NADH accumulated during biomass production[1]
- Under **aerobic conditions**, NADH dehydrodgenases or the Glycerol-3-phosphate shuttle can be used to oxidize redox equivalents[1]
- High glycerol yields observed with SAV expression strain may be linked to the strain's difficulty respiring, resulting in "anaerobic" metabolism

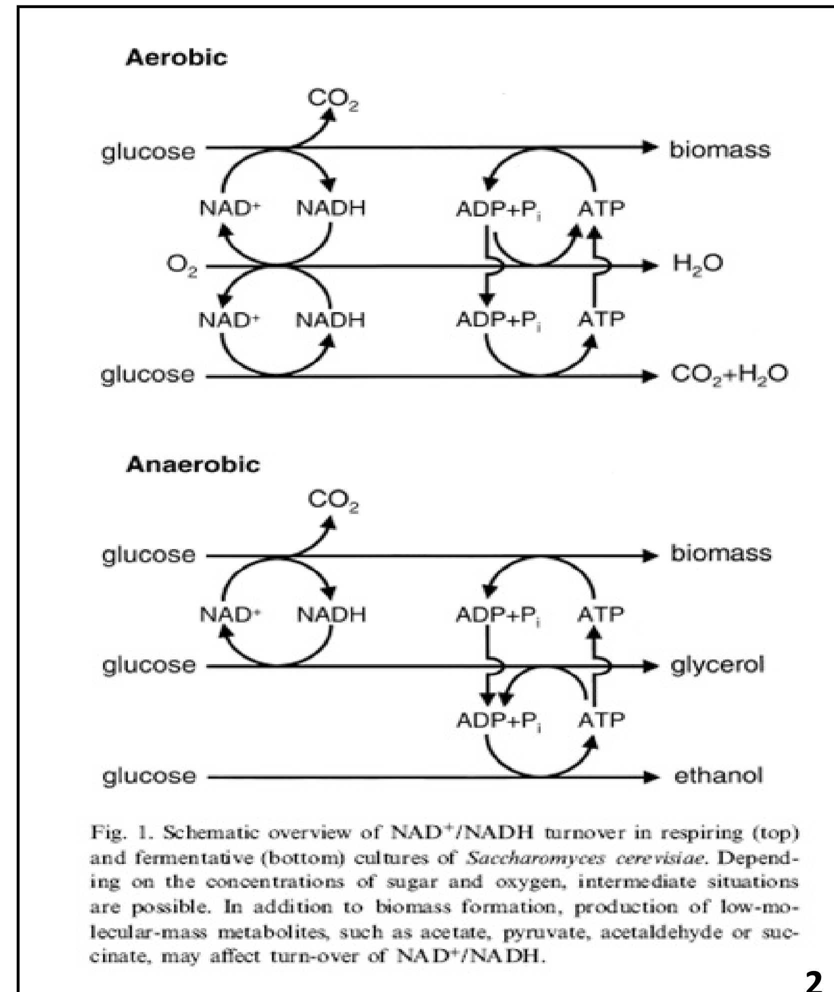
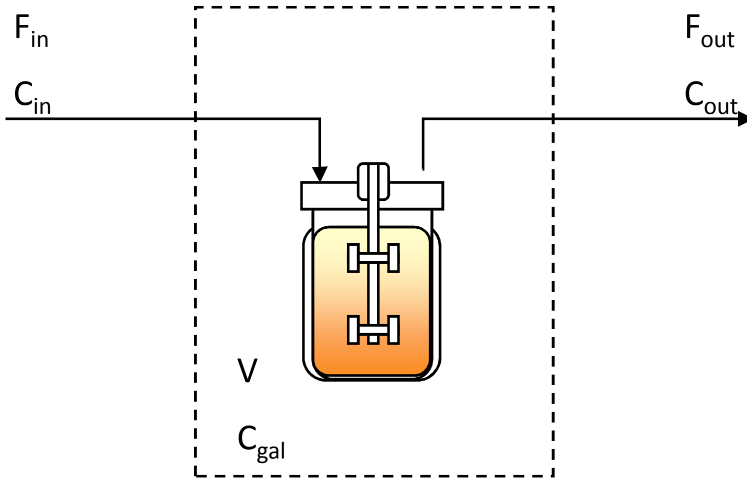
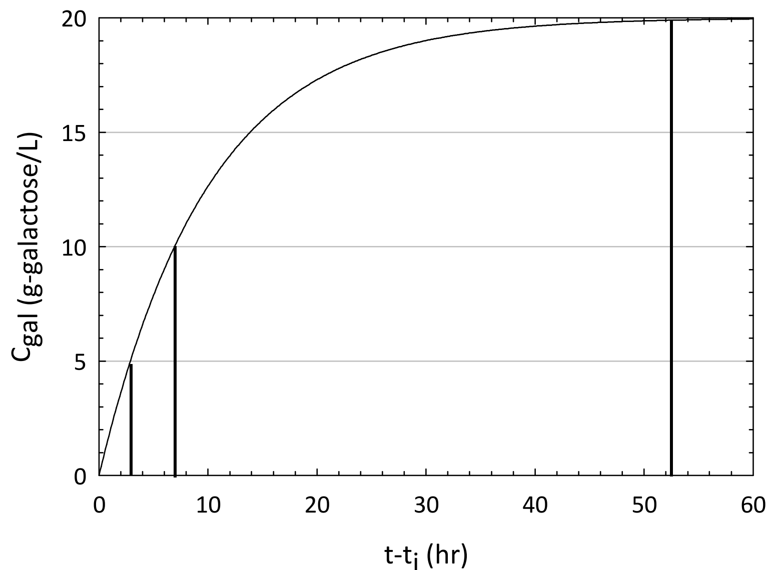


Fig. 1. Schematic overview of NAD^+/NADH turnover in respiring (top) and fermentative (bottom) cultures of *Saccharomyces cerevisiae*. Depending on the concentrations of sugar and oxygen, intermediate situations are possible. In addition to biomass formation, production of low-molecular-mass metabolites, such as acetate, pyruvate, acetaldehyde or succinate, may affect turn-over of NAD^+/NADH.

1. J.-M.A. Geertman et al. Metabolic Engineering 8 (2006) 532-542
2. B.M. Bakker et al. FEMS Microbiology Reviews 25 (2001) 15-37

Galactose Time-Point Calculation





Note: Derivation assumes no galactose consumption

$$\frac{d}{dt}(C_{gal}V) = F_{in}C_{in} - F_{out}C_{out}$$

$$F_{in} = F_{out} = F$$

$$C_{out} = C_{gal}$$

$$\frac{d(C_{gal})}{dt}V + \frac{d(V)}{dt}C_{gal} = F(C_{in} - C_{gal})$$

$$\frac{d(V)}{dt} = 0$$

$$\frac{d(C_{gal})}{dt} = \frac{F}{V}(C_{in} - C_{gal})$$

$$\frac{d(C_{gal})}{(C_{in} - C_{gal})} = \frac{F}{V}dt$$

$$\int_{C_{gal,i}}^{C_{gal}} \frac{d(C_{gal})}{(C_{gal} - C_{in})} = -\int_{t_i}^{t} \frac{F}{V}dt$$

$$C_{gal,i} = 0$$

$$ln(C_{gal} - C_{in})\Big|_0^{C_{gal}} = -\frac{F}{V}t\Big|_{t_i}^{t}$$

$$ln(C_{gal} - C_{in}) - ln(-C_{in}) = -\frac{F}{V}(t - t_i)$$

$$ln(C_{gal} - C_{in}) = ln(-C_{in}) - \frac{F}{V}(t - t_i)$$

$$C_{gal} - C_{in} = -C_{in}e^{-\frac{F}{V}(t-t_i)}$$

$$C_{gal}(t) = C_{in}\left(1 - e^{-\frac{F}{V}(t-t_i)}\right)$$

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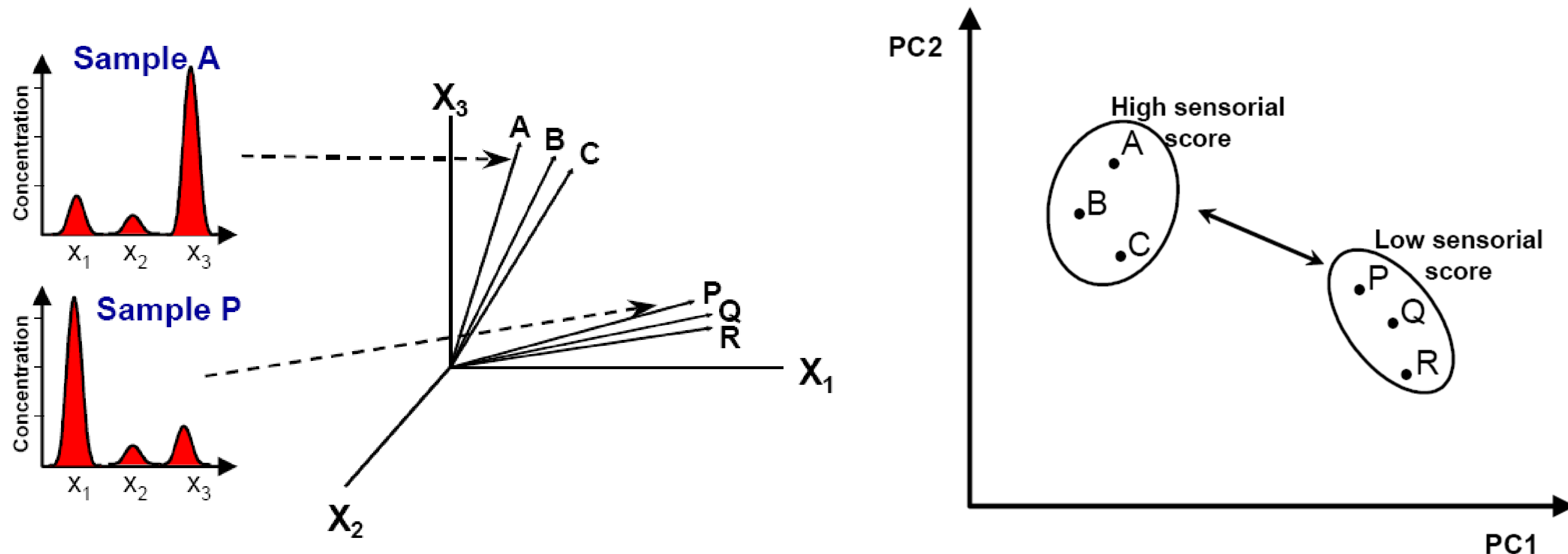
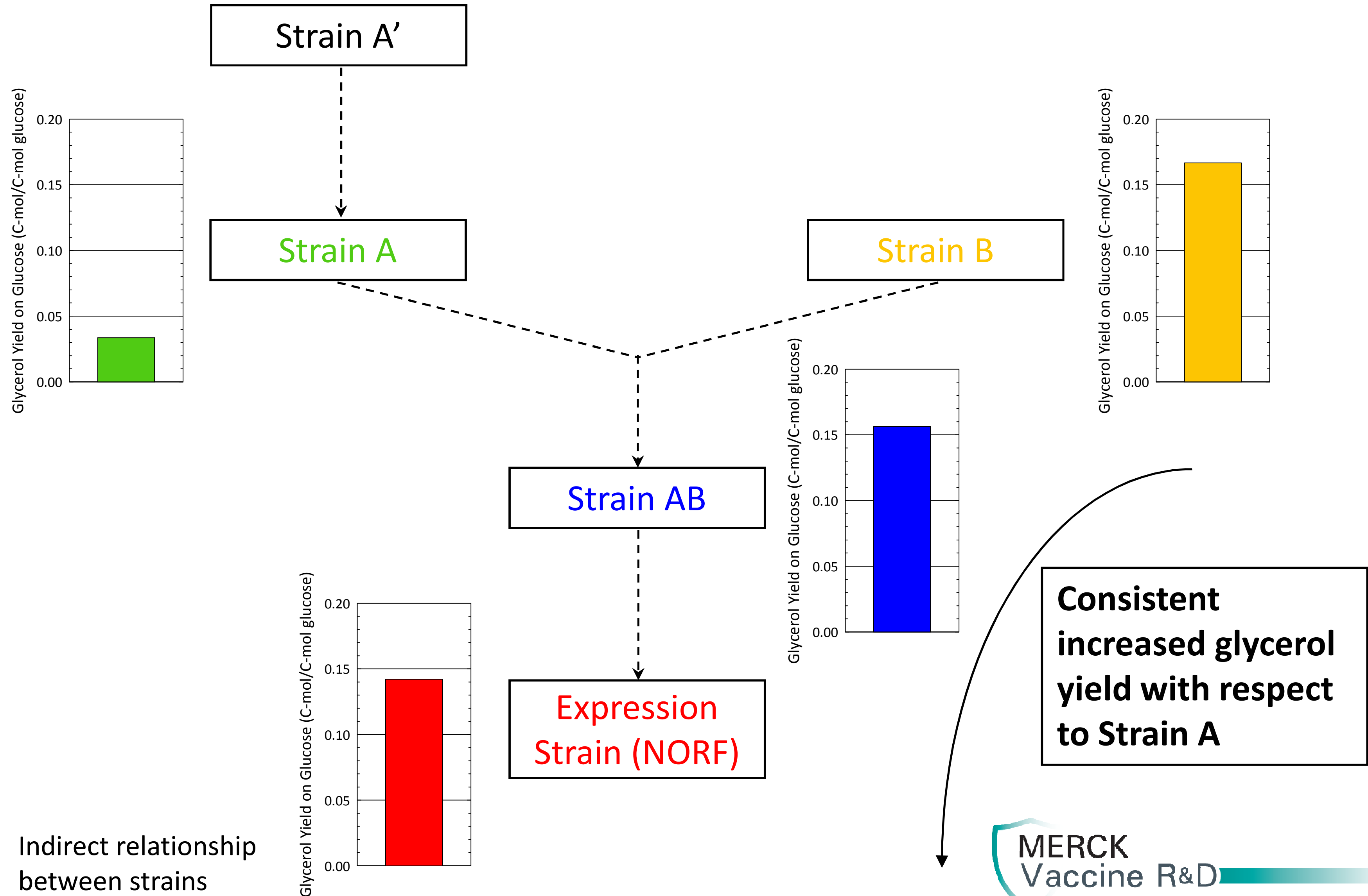
Principle Component Analysis

- Data sets composed of *n* variables are converted into an *n*-dimensional space
  - The human mind can't interpret data sets visualized in multi-hundred or multi-thousand dimensional spaces
  - Therefore, it is necessary to project an *n*-dimensional space into a 2- or 3-dimensional space
- PCA concentrates strongly correlating variables, i.e. variables that vary in a similar way in all data sets, into a new variable
- This new variable, the principal component (PC) is a linear combination of the original variables
- PCA aims at establishing relationships between the *m* rows (biological samples) and *n* columns (variables, e.g. gene expression levels or metabolite concentrations) of a matrix (dimension *m* x *n*)
- A plot can be drawn of two PC's which allows the similarity of samples to be visualized

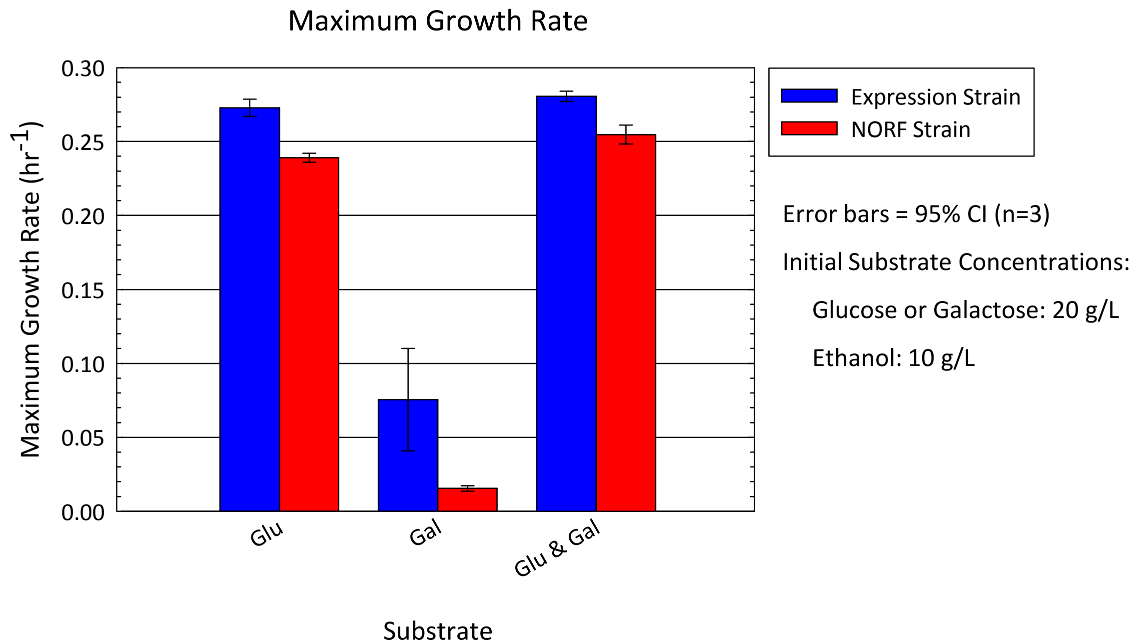


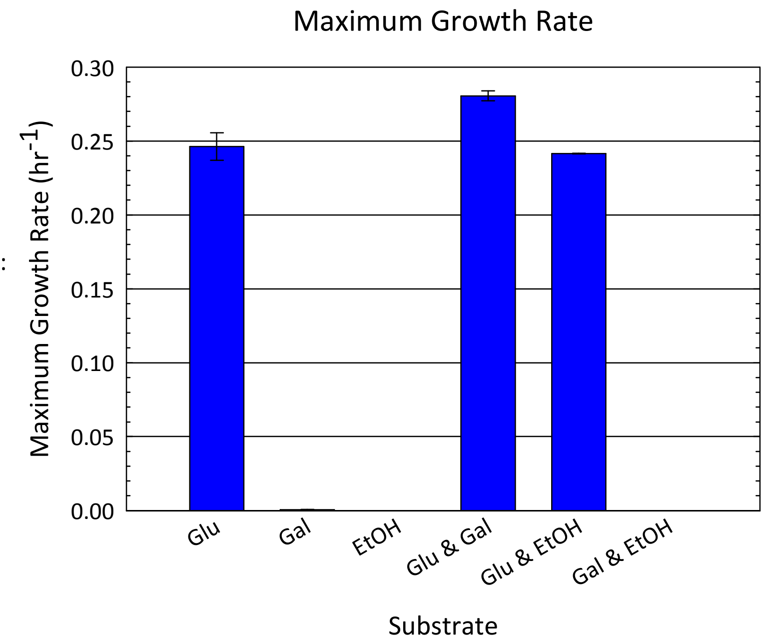
Adapted from TNO Innovation for Life proposal: Exo-metabolomics of biopharmaceutical protein-producing *S. cerevisiae* and *P. pastoris*, 2009.

Glycerol Yield of Parental Strains



Process Understanding Shake Flask Experiments: Growth Evaluation on Various Carbon Sources





Leaky expression was not found in the absence of galactose; galactose uptake must be occurring in order to promote transcription of the antigen gene (data not shown).

The lack of growth on ethanol as a sole carbon source supports the hypothesis that the expression strain has a general respiratory deficiency, not just a galactose pathway dysfunction

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