Development of a general defined media for *Pichia pastoris* protein expression

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An increasing fraction of new medicines are recombinant protein therapeutics

Over 200 products approved in past 20 years

Protein Data Bank

Monoclonal antibodies
\(~150\text{kDa}\)

Enzymes
20-50\text{kDa}
These proteins are made using cells

Design gene from protein

- Protein
- RNA
- DNA

Incorporate into cell’s genome

- Chinese hamster ovary (CHO)

Cells express the foreign protein and secrete it into the culture broth

Current CHO-based production process has a few challenges

- Raw materials are expensive
- Production runs are long (2 weeks+)
- Foreign gene integration is complex

Adapted from Birch and Racher, Advanced Drug Delivery Reviews (2006)
**Pichia pastoris** holds great potential for manufacturing of biologic drugs

- Fast growth to high cell density
- Small genome – 4 chromosomes

![Graph showing growth rates of Pichia pastoris and CHO cells](image)

**However, rates of production (titers) are typically lower than those achieved with CHO cells**


Love et al., *BMC Genomics* (2016)

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**Upstream process development toolbox has 3 main components**

- **Strain engineering**
  - Clone screening
  - Targeted mutations

- **Bioreactor parameters**
  - Temperature
  - Stir rate
  - Dissolved O₂

- **Media formulation**
  - Nutrients (C, N, P, S)
  - Feeding strategy

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**Easy to understand visual that separates a list spatially**
Media for *Pichia* has not been studied as extensively as for CHO

<table>
<thead>
<tr>
<th>Media type</th>
<th>CHO</th>
<th>Pichia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex</td>
<td>Fetal bovine serum</td>
<td>Buffered complex medium (BMGY)</td>
</tr>
<tr>
<td>Defined</td>
<td>Carbon source, salts, proteins, hormones, vitamins, amino acids, lipids, etc.</td>
<td>Carbon source, salts, trace elements</td>
</tr>
<tr>
<td></td>
<td>Examples: Ham’s F-12, proprietary formulations</td>
<td>Examples: BSM, FM22, d’Anjou</td>
</tr>
</tbody>
</table>

Here it would be a little neater if the table entries were top aligned with each other!

Approach and methods

Our goal was to design a defined media that reduced the metabolic burden on the organism, evaluated by growth rate

*Slide offsets the main goal (top) from less critical information (below)*

We integrated three strategies

- Systematic screening to understand limitations of current media and identify nutrient supplements
- Analytical methods to identify nutrients and tune concentrations
- Transcriptomics for deeper view of biological processes
Reminder: idealized growth phases for batch fermentation

For *Pichia*, growth in basal salts media is significantly slower than in complex media

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10mL microtiter plates
Bartlett et al., manuscript in preparation

**Something in the basal salts medium was inhibiting growth**
Reducing ammonium concentration increased exponential growth rate to 0.17h⁻¹

Looking for further improvement, we tried other salts formulations

FM22 and d’Anjou medium have both been used for *Pichia* fermentations

- Lower salt content than BSM

For comparison, set NH₄⁺ concentrations of all to 25mM

Used d’Anjou medium with 25mM NH₄⁺ as base for further optimization
We used knowledge about complex media to select defined components for screening.

Stock solutions of some nutrients have previously been tried:

- Vitamins
- Nucleosides

Carbohydrate concentrations in yeast extract have been measured:

- Lactate: up to 10mM
- Trehalose: up to 5mM

From HPLC:

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>4.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.7</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.1</td>
</tr>
<tr>
<td>Serine</td>
<td>0.8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.3</td>
</tr>
<tr>
<td>Total</td>
<td>22.8</td>
</tr>
</tbody>
</table>

Bartlett et al., manuscript in preparation

Glutamine, arginine, and vitamins had the greatest impact on growth rate.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Concentration</th>
<th>μ (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex media</td>
<td>-</td>
<td>0.248 ± 0.001</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>0.196 ± 0.001</td>
</tr>
<tr>
<td>Glutamine</td>
<td>5mM</td>
<td>0.217 ± 0.001</td>
</tr>
<tr>
<td>Vitamins</td>
<td>1x</td>
<td>0.204 ± 0.001</td>
</tr>
<tr>
<td>Arginine</td>
<td>5mM</td>
<td>0.202 ± 0.001</td>
</tr>
<tr>
<td>Lysine</td>
<td>5mM</td>
<td>0.196 ± 0.002</td>
</tr>
<tr>
<td>Nucleotides</td>
<td>1x</td>
<td>0.196 ± 0.002</td>
</tr>
<tr>
<td>Alanine</td>
<td>5mM</td>
<td>0.195 ± 0.002</td>
</tr>
<tr>
<td>Trehalose</td>
<td>5mM</td>
<td>0.193 ± 0.001</td>
</tr>
<tr>
<td>Lactate</td>
<td>10mM</td>
<td>0.186 ± 0.002</td>
</tr>
</tbody>
</table>

Bartlett et al., manuscript in preparation

Voroby et al., Yeast (1992)

Great use of a box or color change to highlight important details.
Growth in Generation 1 medium was comparable to BMGY during exponential phase, then leveled off.

In Gen 1 medium, NH\(_4^+\) was sufficient but amino acids were fully consumed.

White space lets the data be seen clearly.

Nice side by side comparison where the author has been sure to make the graphs consistent with each other.
To further characterize metabolic differences, we performed RNA-Seq

**Output:** Prepares the audience for the type of data they will see

<table>
<thead>
<tr>
<th>Gene</th>
<th>BMGY</th>
<th>d'Anjou</th>
<th>Gen 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARO10</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>POX1</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>CAR1</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>POT1</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>GDH3</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>CAR2</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>COX15</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>SPS4</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>FLO9</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>PUT1</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>...</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
</tbody>
</table>

Data is analyzed by comparing gene expression between conditions

Computational methods have been developed for different levels of comparison:
- Individual genes
- Pathways or gene sets

Reporter metabolites method was used to identify expression differences at pathway level

This method has not previously been used for media design

Known difference in vitamin metabolism was visible in the transcriptome

We used the same approach to identify other areas with significantly different metabolism

The most significantly different metabolites for both defined formulations are involved in fatty acid oxidation

Fatty acids are present in BMGY but not in either defined formulation

Implication: try adding fatty acids

Bartlett et al., manuscript in preparation
Fatty acids and increases to amino acid concentrations improved performance

Color reinforces the information

hGH productivity was ~10x higher in Gen 2 medium than BMGY or BSM

<table>
<thead>
<tr>
<th>Media</th>
<th>BMGY</th>
<th>BSM</th>
<th>Gen 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass after outgrowth (OD600)</td>
<td>14.3</td>
<td>9.03</td>
<td>17.9</td>
</tr>
<tr>
<td>Biomass after induction (OD600)</td>
<td>23.5</td>
<td>15.4</td>
<td>23.1</td>
</tr>
<tr>
<td>Titer by GX (mg/L)</td>
<td>22.1</td>
<td>&lt;LOD</td>
<td>201</td>
</tr>
</tbody>
</table>

We see parallel structure for the table and raw data which is intuitive for us to understand
G-CSF productivity in bioreactors was also higher in Gen 2 medium than BMGY

Summary of results

We developed a defined media for *Pichia pastoris* that supported cell growth at the same rate as in BMGY and led to higher protein productivity

We identified metabolic gaps and addressed them through transcriptomics, analytical methods, and systematic screening

Future work will focus on optimizing Generation 2 media specifically for productivity
Implications

<table>
<thead>
<tr>
<th>Transcriptomic analysis is powerful</th>
<th>Productivity in <em>Pichia</em> will increase</th>
<th>Biologic manufacturing costs are addressable</th>
</tr>
</thead>
</table>

Productivity in *Pichia* will increase.

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Questions?