Carbon and energy distribution through propagation and fermentation: keeping your yeast on track

Kelly Hahler
Turning science into **industrial reality**

We are the business unit of Lesaffre, focusing on the worldwide development and sales of value-added fermentation solutions for fuel ethanol and bio-based chemicals producers.

Our mission:

1. **1G**: Offer value added fermentation products to the first generation fuel ethanol industry

2. **2G**: Continue innovating in the field of lignocellulosic ethanol.

3. Develop economically viable solutions for bio-based chemicals producers.
Our products

**Ethanol Red®**
#1 yeast among first generation ethanol producers, Ethanol Red® tolerates high ethanol levels and is ideal for the production of industrial ethanol from starch with a final ethanol concentration of up to 18% (v/v).

**Safdistil™ Plus**
Hybrid yeast selected for its superior fermentation kinetic, temperature resistance and alcohol tolerance. Recommended on both sugar (thick juices of various compositions) and starch substrates.

**Safdistil™ C-70**
A very robust yeast strain capable of fermenting various substrates (molasses, C-starch, grains) at different temperatures from 25C to 35C.

**Propaide™**
Balanced yeast nutrient complex based on the synergies of organic, mineral and vitamin’s growth factors. Its formula was specifically developed to increase industrial fermentation of grain mashes and enhance yeast growth & metabolic kinetics.

**Cellux™**
Cellux™ is Leaf Technologies selected yeast for lignocellulosic fuel ethanol production.
Agenda

1. Carbon cycle
2. Yield
3. Mass and energy conservation
4. Yeast metabolism pathways
5. How stress diverts carbon, reducing yield
Carbon cycle in biofuel production
Yield: carbon from starch to ethanol

\[(C_6H_{10}O_5)_n + H_2O \xrightarrow{H^+ \text{ or enzyme}} C_6H_{12}O_6 \xrightarrow{\text{yeast}} 2C_2H_5OH + 2CO_2\]

Maximum Ethanol

Minimum Inputs

1. Feedstock (corn)
2. Energy
3. Time
Yield: tracking carbon from starch to ethanol

### Proximate Analysis of Corn Grain

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Rangea</th>
<th>Averageb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%, wet basis)</td>
<td>7–23</td>
<td>16.0</td>
</tr>
<tr>
<td>Starch (%, dry basis)</td>
<td>61–78</td>
<td>71.7</td>
</tr>
<tr>
<td>Protein (%, dry basis)</td>
<td>6–12</td>
<td>9.5</td>
</tr>
<tr>
<td>Fat (%, dry basis)</td>
<td>3.1–5.7</td>
<td>4.3</td>
</tr>
<tr>
<td>Ash (oxide) (%, dry basis)</td>
<td>1.1–3.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Pentosans (as xylose) (%, dry basis)</td>
<td>5.8–6.6</td>
<td>6.2</td>
</tr>
<tr>
<td>Fiber (neutral detergent residue) (%)</td>
<td>8.3–11.9</td>
<td>9.5</td>
</tr>
<tr>
<td>Cellulose + lignin (acid detergent residue) (%)</td>
<td>3.3–4.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Sugars, total (as glucose) (%, dry basis)</td>
<td>1.0–3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Total carotenoids (mg/kg)</td>
<td>5–40</td>
<td>30</td>
</tr>
</tbody>
</table>
Yield: tracking carbon from starch to ethanol

\[ 56 \times \text{starch} \times \text{moisture} \div 11.59 = \text{theoretical yield} \]

\[ 56 \times 0.72 \times 0.85 \div 11.59 = 2.96 \]
\[ 56 \times 0.70 \times 0.85 \div 11.59 = 2.87 \]

Importance of feedstock starch on yield
Yield: tracking carbon from starch to ethanol

The **law of conservation of mass** tells us that matter cannot be created or destroyed.

Chemical equations must be balanced:

\[ \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2 \text{CH}_3\text{CH}_2\text{OH} + 2 \text{CO}_2 \]

- glucose  
- ethanol  
- carbon dioxide
Carbon storage in corn through photosynthesis

Amylose

Amylopectin
Carbon pathway in yeast
Aerobic and anaerobic metabolism

Aerobic
1. Glycolysis
2. Kreb’s Cycle
3. Electron Transport Chain

Anaerobic
1. Glycolysis
2. Fermentation
Carbon through glycolysis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Role</th>
<th>Steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinase</td>
<td>Transfers phosphate</td>
<td>1,3,7,10</td>
</tr>
<tr>
<td>Isomerase</td>
<td>Converts to isomer</td>
<td>2,5</td>
</tr>
<tr>
<td>Aldolase</td>
<td>Aldol reaction</td>
<td>4</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>Removes hydrogen</td>
<td>6</td>
</tr>
<tr>
<td>Mutase</td>
<td>Shifts a functional group</td>
<td>8</td>
</tr>
<tr>
<td>Enolase</td>
<td>Removes a molecule of water</td>
<td>9</td>
</tr>
</tbody>
</table>
Carbon through aerobic or anaerobic metabolism
Carbon distribution in ideal conditions

95% = ethanol + carbon dioxide

1% = new yeast cells

4% = other end products

![Chemical structures of pyruvate, acetaldehyde, glycerol, acetate, and lactate.](Image)
Carbon distribution disruptions

- **GLUCOSE**
  - Glucose - 6 - phosphate
  - Fructose 1,6 - bisphosphate
  - Glyceraldehyde - 3 - phosphate
  - Dihydroxyacetone phosphate

- **Residual Starch**
  - Nucleotides
  - Storage carbohydrates
  - Sugar - Nucleotides
  - Structural polysaccharides

- **Glycerol**
  - Lipids
  - Fatty acids

- **Acetaldehyde**
  - ETHANOL
  - Acetate

- **Fusels**
  - TCA Cycle
  - Amino acids
  - Oxaloacetate
  - α-Ketoglutarate
  - Succinate
  - Cytochromes

- **Acetate**

Leaf
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A LESAFFRE BUSINESS UNIT
Chemical energy: Propagation and fermentation mass balance

**Propagation**

\[ \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 \rightarrow 6\text{CO}_2 + 6\text{H}_2\text{O} + 686 \text{ Kcal Energy} \]

- **180g** 192g 264 g 108 g

**Fermentation**

\[ \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{C}_2\text{H}_5\text{OH} + 2\text{CO}_2 + 45 \text{ Kcal Energy} \]

- **180g** 92g 88g

**Theoretical yield** = 92g/180g = \textbf{0.51 g ethanol g}^{-1} \text{ glucose}

**Practical yield** = \textbf{0.46 g/g} = 82.8 g ethanol \text{ mol}^{-1} \text{ glucose}

10% losses = maintenance energy + byproducts
Chemical energy from propagation = ATP (chemical) + metabolic heat

Only ~40% of the 686 kcal equivalent in glucose is converted into ATP

Propagation

Glucose 686Kcal $\rightarrow$ 274 Kcal (~30 ATP) + 412 kCal metabolic heat

Therefore, Energy/ATP = 274/30 = 9.1 kCal

Metabolic heat: Heat generated by cells due to thermodynamic efficiency limitations of living cells
Chemical energy from fermentation = ATP (chemical) + metabolic heat

Only ~40% of the 45 kcal equivalent in glucose is converted into ATP

Fermentation

Glucose 45Kcal → 18 Kcal (2 ATP) + 27 kCal metabolic heat

Therefore, Energy/ATP = 18/2 = 9 kCal

Metabolic heat: Heat generated by cells due to thermodynamic efficiency limitations of living cells
Propagation is where the largest amount of ATP is produced and stored.

Yeast are "charging" their battery.
Powering a 40W light bulb using the ATP produced through the pathways

All calculations for 1 mole (180 g) glucose at theoretical yields

1 kcal = 1.163 Wh

Aerobic = 686 kcal x 40% ATP energy x 1.163 Wh/kcal = 319 Wh

Anaerobic = 45 kcal x 40% ATP energy x 1.163 = 21 Wh

Fermentation 31 minutes

Respiration 8 hrs
Powering a 40W light bulb using the metabolic heat produced through the pathways

All calculations for 1 mole (180 g) glucose at theoretical yields

1 kcal = 1.163 Wh
Aerobic = 686 kcal x 60% heat x 1.163 Wh/kcal = 479 Wh
Anaerobic = 45 kcal x 60% x 1.163 = 27 Wh

Fermentation

36 minutes

Respiration

12 hrs
Powering a 40W light bulb using the ethanol produced through the pathways

All calculations for 1 mole (180 g) glucose at theoretical yields

1 kcal = 1.163 Wh, 1g ethanol = 7 kcal

Aerobic = 686 kcal x 0g ethanol x 1.163 Wh/kcal = 0 Wh

Anaerobic = 92g ethanol x 7 kcal/g x 1.163 = 749 Wh

Fermentation

18 hrs 52 minutes

Respiration

0 minutes
Propagation or fermentation: Net energy is the same

Energy can neither be created nor destroyed; rather, it is transformed from one form to another
(first law of thermodynamics)

<table>
<thead>
<tr>
<th>Energy form</th>
<th>Respiration 40W bulb time</th>
<th>Fermentation 40W bulb time</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>8 hrs</td>
<td>32</td>
</tr>
<tr>
<td>Metabolic heat</td>
<td>12 hrs</td>
<td>36</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0</td>
<td>18 hr 52 min</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20 hrs</strong></td>
<td><strong>20 hrs</strong></td>
</tr>
</tbody>
</table>
Fermentation Stresses

- **Biological Stressors**
  - bacteria
  - wild yeast
  - ageing
  - mutation

- **Physical Stressors**
  - temperature
  - osmotic pressure
  - pressure

- **Chemical Stressors**
  - organic acids
  - pH
  - toxic compounds
  - nutrition lack
  - ethanol

**Yeast Stress**
Environmental Stress Response (ESR)

~900 genes that alter expression in response to stress

Heat stress  Acid stress  Ethanol stress  Oxid've stress

Genome
Where stress occurs in your process

Temperature, hydrostatic pressure, fusels, inhibitors

Oxidative

Osmotic

Acid

Ethanol/nutrient

![Graph showing substrate/product (wt%) vs. time (hrs)](image)

- Glucose
- Ethanol

Substrate/product (wt%) vs. Time (hrs)
Increased ATP requirements during stress

Stressed cells use ~8mmol ATP/g cell versus 2 mmol ATP/g normal cells
Impact of 4X ATP burden on ethanol yield

<table>
<thead>
<tr>
<th>Process Condition</th>
<th>Substrate</th>
<th>Product 1</th>
<th>Product 2</th>
<th>Product 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation stoichiometry</td>
<td>1 mole glucose</td>
<td>2 moles ethanol</td>
<td>2 ATP</td>
<td>Byproducts</td>
</tr>
<tr>
<td>Normal (kCal)</td>
<td>686</td>
<td>641</td>
<td>15.2</td>
<td>30 kcal metabolic heat</td>
</tr>
<tr>
<td>Heat shock (kCal)</td>
<td>686</td>
<td>589.4</td>
<td>60.8</td>
<td>30</td>
</tr>
</tbody>
</table>

1. Productivity losses = 589.4/641 = 9.5%
2. A plant producing 14 wt% will only produce 13 wt% under heat stress because of carbon losses in ATP (4X higher maintenance energy) + higher glycerol production
Metabolic heat production in a 16,000 gal prop

<table>
<thead>
<tr>
<th>Propagation tank</th>
<th>Time 0 hr</th>
<th>Time 8 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>50 g/L</td>
<td>10 g/L</td>
</tr>
<tr>
<td>Glucose consumed</td>
<td>0</td>
<td>40 g/L</td>
</tr>
<tr>
<td>Moles</td>
<td>0</td>
<td>40/180 = 0.222</td>
</tr>
<tr>
<td>Metabolic heat (412 kcal/mol)</td>
<td>91.56 kcal/L</td>
<td></td>
</tr>
<tr>
<td>Prop size (16,000 gal)</td>
<td>60,500L</td>
<td>60,500L</td>
</tr>
<tr>
<td>Metabolic heat (kcal)</td>
<td>553,7549 kcal</td>
<td></td>
</tr>
<tr>
<td>Total heat kWh (Kilowatt hr)</td>
<td>805 kWh</td>
<td></td>
</tr>
</tbody>
</table>

1. Metabolic heat is enough to cool an average Midwest 2000 sq ft house in summer for 1 full month (~800 kWh electricity)
2. Enough energy to raise prop temperature by ~2°C or 35°F
Carbon expense of heat stress

1. Reserve ATP (battery) is used to
   a) Synthesize stress protection proteins
   b) Repair damaged DNA, reversibly denatured proteins

2. Glucose is redirected to glycerol, trehalose and glycogen reducing ethanol productivity

3. A single heat stress event (100°F for 2 hrs) can reduce yield by as much as 10%

4. Micronutrients play key role in stress protection
Carbon expenditure:
ESR induces both trehalose synthase and trehalase

Trehalose synthesis

**Glucose** ➔ Trehalose (trehalose synthase), Optimum T 104°F

Trehalose breakdown

Trehalose ➔ Glucose (trehalase), Optimum T 77°F

trehalose reutilization (conversion to glucose) is slow because of higher temperatures employed in fermentation
Carbon expense of heat stress
Heat shock (100°F) increases DP2 and DP4+

Increased glycogen synthesis
Increased trehalose synthesis

Leftover sugars (wt %)

ER
ER + heat stress

Glucose DP2
DP3 DP4+
Conserving carbon: Yeast morphology

1. Normal cell – 2 or 3 large vacuoles
2. Elongated cell – Mg, N₂ limitation
3. No cell definition – Zn deficiency, propagation issues (sterols)
4. Vacuole fragmentation – Osmotic stress (excess glucose), salt stress (excess Na, K) oxidative stress (excess Fe), Mn, Cu or Zn deficiency (low SOD activity)
5. Elongated cells with pseudo-hyphal chains – N₂ deficiency
Carbon conservation: Using nutrition to mitigate stress

• Zinc- protects against oxidative stress in cytoplasm

• Magnesium- promotes growth, cell division and required in stress response pathways

• Potassium- protects against acid & osmotic stress

• Copper- protects against oxidative stress and required in stress response pathways

• Manganese- essential for oxidative stress protection in mitochondria
Carbon conservation:
Using nutrition to mitigate stress

Figure 2: Influence of minerals on ethanol, residual sugars, glycerol and acetaldehyde produced by strain Ethanol Red (ER)
Carbon expenditure: Nitrogen dose

Excess (600 ppm or higher)
- **Glucose** converted to biomass (instead of ethanol)
- Increased contamination (because of higher N₂ availability)
- Increased fermentation rate, metabolic heat and temperature issues
- Higher fusels (if N₂ comes from FAN)

Deficiency (200 ppm or less)
- Stuck or sluggish fermentation
- Leftover sugars if fermentation isn’t completed in time allotted
- Elongated cells
- Poor stress tolerance
- Increased **trehalose** content, higher oxidative stress
Carbon expenditure: Competition from bacteria

**Effect of infection on yield**

<table>
<thead>
<tr>
<th>Infection level (bacteria/ml)</th>
<th>Loss in yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1 million</td>
<td>Up to 1%</td>
</tr>
<tr>
<td>1-10 million</td>
<td>1-3%</td>
</tr>
<tr>
<td>10-100 million</td>
<td>3-5%</td>
</tr>
<tr>
<td>Over 100 million</td>
<td>Over 5%</td>
</tr>
</tbody>
</table>

*Lactic acid inhibition > 0.2% w/w  
Acetic acid inhibition > 0.05% w/w*

**Bacteria Growth: 4-6 times faster than Saccharomyces:**
- Substrate competition-redirection: less glucose & nutrients for yeast
- Organic acids production is a loss in ethanol yield
Acetic acid decreases sugar consumption and increases lag phase

<table>
<thead>
<tr>
<th></th>
<th>S. cerevisiae</th>
<th>Z. bailii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>9 g L⁻¹ acetic acid</td>
</tr>
<tr>
<td><strong>Specific production/consumption rate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>μ̄max, Glucose phase</td>
<td>0.44±0.01</td>
<td>0.30±0.00</td>
</tr>
<tr>
<td>μ̄max, Acetic acid phase</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>qGlucose</td>
<td>cmol×cmol DW⁻¹×h⁻¹</td>
<td>-2.87±0.02</td>
</tr>
<tr>
<td>qAcetic acid</td>
<td>cmol×cmol DW⁻¹×h⁻¹</td>
<td>0.05±0.00</td>
</tr>
<tr>
<td><strong>Yield from total substrate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yₓ/s</td>
<td>cmol×cmol⁻¹</td>
<td>0.15±0.00</td>
</tr>
<tr>
<td>YₑtOH/s</td>
<td>cmol×cmol⁻¹</td>
<td>0.45±0.01</td>
</tr>
<tr>
<td>YAcetic acid/s</td>
<td>cmol×cmol⁻¹</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td>YGlycerol/s</td>
<td>cmol×cmol⁻¹</td>
<td>0.03±0.00</td>
</tr>
<tr>
<td>YPyruvate/s</td>
<td>cmol×cmol⁻¹</td>
<td>0.004±0.000</td>
</tr>
<tr>
<td>Y Succinate/s</td>
<td>cmol×cmol⁻¹</td>
<td>0.000±0.001</td>
</tr>
<tr>
<td><strong>Lag phase before initial growth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag phase</td>
<td>h</td>
<td>0.25±0.05</td>
</tr>
</tbody>
</table>

*S. cerevisiae* and *Z. bailii* were cultured in minimal medium using bioreactors. Different amounts of acetic acid were added to the medium to stress the microorganisms equally. The results were calculated from at least three biological replicates, and are given as the means ± standard deviation.

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Carbon expenditure on fusel production

Fusels originate from amino acids and are normal metabolites for yeast

Fusels help maintain redox balance in cells

Glucose to Biomass

NAD+ (oxidized)

Amino acid pool (AA)

AA reduction (fusels)

Protein Synthesis

Protein

Protein

NADH (reduced)

Ethanol

Growth

Stress protection

Leaf

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What triggers higher fusel production?

Fusels help maintain redox balance in cells

Glucose to Biomass

Higher glucose in prop: Excess NADH

NAD+ (oxidized)

NADH (reduced)

High amino nitrogen

AA to fusels

Stationary phase: Tyrosol & Tryptophanol signal cell death (sensing molecules)
Carbon expenditure on glycerol: Physiological roles of glycerol

1. **Stress protection**
   a. Osmotic stress (high transient sugars, salts)
   b. Ethanol tolerance, heat tolerance, oxidative stress

2. **Maintenance of cellular integrity**
   a. Required for drying yeast
   b. Protection of cell (viability) during rehydration

3. **Redox balance**
   a. Primary pathway for conversion of excess NADH (generated during growth) back to NAD+
Redox balance

Oxidation reaction (respiration, propagation)

\[ \text{Reductant} \rightarrow \text{Product} + e^- \]

Oxidation is loss of electrons

Reduction reaction (glycerol formation)

\[ \text{Oxidant} + e^- \rightarrow \text{Product} \]

Reduction is gain of electrons

Glycerol is an important electron acceptor in anaerobic growth. In aerobic growth the final electron acceptor is \( O_2 \).
Loss of Electrons is Oxidation (LEO)
Gain of Electrons is Reduction (GER)
Key aspects of redox balance

1. Typically, an oxidation is always coupled with a reduction.

2. If a preferred reduction pathway (glycerol) is compromised, cells will overproduce some other product (acetic acid, fusels) until cell is redox neutral (homeostasis). This slows growth in fermentation and uses **carbon**.

*Low glycerol strains rely on alternate electron acceptors. These modifications use less carbon and provide yield benefits without overproducing acetate/fusels.*
Glycerol helps in cofactor (NADH) recycling

Glucose

Biomass

NADH (red)

NAD+ (ox)

Glycerol, Ethanol, Fusels, Acetate

Oxidative

< Redox balance >

Reductive
Summary

1. Mass and energy cannot be created or destroyed
2. Ideal carbon to ethanol and carbon dioxide is 95%
3. Glucose is needed to generate ATP
4. ATP demand is higher during stress response
5. ESR causes expenditure of carbon and energy
6. Nutrition assists in stress protection
Dinner at the Yeastwood’s

Ouch, I am so sore...

Ethanol

Glucose

Ammonia

Crabtree Ball 2016
Increasing CO₂ emissions one yeast at a time

Is this what …sic…they call a sugar high?

I am fully wasted..