Optimizing hop aroma in beer dry hopped with Cascade utilizing glycosidic enzymes

Young Scientist Symposium – Chico, CA 2016
Kaylyn Kirkpatrick
New Belgium Brewing Co.
US craft beer hopping rates and usage

*Source: Brewers Association*
US trending towards aroma varieties

From 2012 – 2013...
• USA Aroma increased **28.53%**
• USA Alpha increased 1.28%
• US aroma/alpha acres now number 7/3 (Brewers Association)

*Source: IHGC Economic Commission annual reports*
Cascade – a popular choice

Top Hops | 2007
1. Cascade (Aroma)
2. Centennial (Dual)
3. Willamette (Aroma)
4. Chinook (Dual)
5. Amarillo (Aroma)
6. EKG (East Kent Golding) (Dual)
7. Saaz (Aroma)
8. CTZ | Columbus, Tomahawk, and Zeus (Bittering)
9. U.S. Golding (Aroma)
10. Styrian Golding (Aroma)

Top Hops | 2015
1. Cascade (Aroma)
2. Centennial (Dual)
3. Chinook (Dual)
4. Simcoe® (Aroma)
5. Citra® (Aroma)
6. Hallertau Mittelfruh (Aroma)
7. Amarillo (Aroma)
8. Crystal (Aroma)
9. Magnum (Bittering)
10. CTZ | Columbus, Tomahawk, and Zeus (Bittering)

Source: Brewers Association 2015 Hop Usage Survey

<table>
<thead>
<tr>
<th>Hop Variety</th>
<th>2012 Acreage</th>
<th>2013 Acreage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahtanum™</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cascade</td>
<td>60</td>
<td>61</td>
</tr>
<tr>
<td>Centennial</td>
<td>35</td>
<td>36</td>
</tr>
<tr>
<td>Chinook</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Citra®</td>
<td>37</td>
<td>38</td>
</tr>
<tr>
<td>Fuggle</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Golding</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Hallertau</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Liberty</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Magnum</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Nugget</td>
<td>&lt;1</td>
<td>5</td>
</tr>
<tr>
<td>Palisade®</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Perle</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Simcoe®</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>Sterling</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Summit™</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Willamette</td>
<td>0</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
Exploring hop aroma – brewing industry

• Sustainability
  • How can we make the best use of our natural resources?

• New product development
  • How can we create unique and desirable hop flavor for consumers?

• Process efficiency
  • How can we use the tools that we have to optimize and better control hop utilization in brewing process?
Hop oil fractions

Myrcene
Estimated threshold: ~30 ppb in beer (Hieronymus, 2012)

Linalool
Estimated threshold: ~10 ppb in beer (Almaguer, 2014)

3-sulphanylhexan-1-ol
Estimated threshold: ~60 ng/L (Takoi, 2006)
Glycosides – potential flavor precursors in hops

- Yeast, enzyme or acid treatment
- Aliphatic alcohols, terpene alcohols, phenols, norcarotenoids
- O, S, and N-linked glycosidic bonds
- 41 polyfunctional thiols recently found in hops (Gros, 2013)

## Glycosides in hopped pilsner beer

### Table: Enzymatic hydrolysis of β-glucosidase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Addition of enzyme</th>
<th>Without enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>3(Z)-Hexenol</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>1-Octen-3-ol</td>
<td>484</td>
<td>0</td>
</tr>
<tr>
<td>1,5-Octadien-3-ol</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>Linalool</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>8-Hydroxy-linalool I</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>8-Hydroxy-linalool II</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Benzylalcohol</td>
<td>82</td>
<td>15</td>
</tr>
<tr>
<td>3-Hydroxy-7,8-dihydro-β-ionol</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

Linalool is a key hop aroma compound with strong contribution to “hoppy flavor”

Targeted analysis using SPME GC-MS

• DVB/CAR/PDMS SPME fiber
• Gerstel MPS auto sampler
• Agilent DB-5MS
• Agilent 7890A GC and 5975C MSD
• Compounds targeted via SIM:

<table>
<thead>
<tr>
<th>Relative RT</th>
<th>Myrcene</th>
<th>Limonene</th>
<th>Linalool</th>
<th>2-Undecanone</th>
<th>Geranyl Acetate</th>
<th>Caryophyllene</th>
<th>Humulene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Myrcene 90% technical grade, Acros (R)-(+) L-limonene, Fluka 1.09</td>
<td>1.22</td>
<td>1.56</td>
<td>1.60</td>
<td>1.79</td>
<td>1.85</td>
<td>1.09</td>
</tr>
<tr>
<td>Calibration (ppb)</td>
<td>125 – 1000</td>
<td>125 – 1000</td>
<td>12.5 – 100</td>
<td>12.5 – 100</td>
<td>12.5 – 100</td>
<td>12.5 – 100</td>
<td>12.5 – 100</td>
</tr>
</tbody>
</table>
Non-targeted analysis: Gas Chromatography Olfactory & TOF

• GCO – link to sensory
• Broad view of aroma actives
**Experimental design: phase 1**

**Determine interactions between hop dosing rate and enzyme to optimize aroma compounds**

*Can we access exogenous enzyme to enhance hop aroma and better utilization of dry hop load?*

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>Description</th>
<th>Active ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapidase Hoptimase (HOP)</td>
<td>Aroma precursor extraction in hops</td>
<td>Polygalacturonase (pectinase) &amp; β-glucosidase</td>
</tr>
<tr>
<td>Rapidase Expression Aroma (WG)</td>
<td>Aroma precursor extraction in white grape</td>
<td>Polygalacturonase and N-arabinofuranosidase</td>
</tr>
<tr>
<td>Rapidase Extra Fruit (RG)</td>
<td>Aroma precursor extraction in red grape</td>
<td>Polygalacturonase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Row #</th>
<th>Enzyme</th>
<th>Hopping Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HOP</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>HOP</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>HOP</td>
<td>300</td>
</tr>
<tr>
<td>4</td>
<td>WG</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>WG</td>
<td>200</td>
</tr>
<tr>
<td>6</td>
<td>WG</td>
<td>300</td>
</tr>
<tr>
<td>7</td>
<td>RG</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>RG</td>
<td>200</td>
</tr>
<tr>
<td>9</td>
<td>RG</td>
<td>300</td>
</tr>
</tbody>
</table>

9 conditions x 5 repetitions = 45 dry hop reactions

3 enzymes

3 hopping rates
Experimental design flow: phase 1

<table>
<thead>
<tr>
<th>Component Name</th>
<th>Type</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ei</td>
<td>N</td>
<td>2.82</td>
<td>Deg Plato</td>
</tr>
<tr>
<td>ABV</td>
<td>N</td>
<td>6.25</td>
<td>% ABV</td>
</tr>
<tr>
<td>ABW</td>
<td>N</td>
<td>4.89</td>
<td>% ABW</td>
</tr>
<tr>
<td>RDF</td>
<td>N</td>
<td>66.59</td>
<td>RDF</td>
</tr>
<tr>
<td>SG 20/20</td>
<td>N</td>
<td>1.01025</td>
<td>SG</td>
</tr>
<tr>
<td>pH</td>
<td>N</td>
<td>4.55</td>
<td>pH</td>
</tr>
<tr>
<td>Color</td>
<td>N</td>
<td>37.92</td>
<td>EBC</td>
</tr>
<tr>
<td>E†</td>
<td>N</td>
<td>5.95</td>
<td></td>
</tr>
<tr>
<td>Calories</td>
<td>N</td>
<td>192.07</td>
<td>Calories</td>
</tr>
<tr>
<td>BCOG</td>
<td>N</td>
<td>14.41</td>
<td>Deg Plato</td>
</tr>
</tbody>
</table>

1. Enzyme @ 1000 ppm
   - Rapidase Expression Aroma (WG)
   - Rapidase Extra Fruit (RG)
   - Rapidase Hoptimase (HOP)

2. 400 mL ale base for dry hopping

3. Stirred @ 25°C

4. Incubate for 48 hours; centrifuge

5. Cascade @ 100/200/300 g/hL

Identification, quantification, and data analysis using GC-MS.
PCA results – SPME GC-MS of 7 hop aroma compounds
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<th>Enzyme treatment</th>
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<th>Active ingredients</th>
<th>Observed effects</th>
</tr>
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<tr>
<td>Rapidase Hoptimase (HOP)</td>
<td>Aroma precursor extraction in hops</td>
<td>Polygalacturonase (pectinase) &amp; β-glucosidase</td>
<td>• Increase in linalool with hopping rate, but appears to drop off between 200-300 g/hL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Increase in geranyl acetate with hopping rate, though increase is more pronounced at 300 g/hL</td>
</tr>
<tr>
<td>Rapidase Expression Aroma (WG)</td>
<td>Aroma precursor extraction in white grape</td>
<td>Polygalacturonase and N-arabinofuranosidase</td>
<td>• Increase in linalool with hopping rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Little effect seen on geranyl acetate with hopping rate</td>
</tr>
<tr>
<td>Rapidase Extra Fruit (RG)</td>
<td>Aroma precursor extraction in red grape</td>
<td>Polygalacturonase</td>
<td>• Steep increase in linalool with hopping rate – enzyme may not be saturated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Little effect seen on geranyl acetate with hopping rate</td>
</tr>
</tbody>
</table>

Hoptimase β-glucosidase enzyme activity may enhance geranyl acetate concentration at 300 g/hL hopping rate
<table>
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<th>Description</th>
<th>Active ingredients</th>
<th>Observed effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapidase Hoptimase (HOP)</td>
<td>Aroma precursor extraction in hops</td>
<td>Polygalacturonase (pectinase) &amp; β-glucosidase</td>
<td>• Increase in myrcene with hopping rate, but appears to decrease once reaching 300 g/hL – biotransformation?</td>
</tr>
<tr>
<td>Rapidase Expression Aroma (WG)</td>
<td>Aroma precursor extraction in white grape</td>
<td>Polygalacturonase and N-arabinofuranosidase</td>
<td>• Little observed effect – larger increase in concentration between 200-300 g/hL than 100-200 g/hL</td>
</tr>
<tr>
<td>Rapidase Extra Fruit (RG)</td>
<td>Aroma precursor extraction in red grape</td>
<td>Polygalacturonase</td>
<td>• Steep increase in myrcene with hopping rate – enzyme may not be saturated</td>
</tr>
</tbody>
</table>

Hoptimase β-glucosidase activity may cause myrcene concentration to decrease at higher hopping rates
Experimental design flow: phase 2

- Control
  - No enzyme
  - Cascade 250 g/hL
  - 65 hours @ 15°C

- Test
  - 1000 ppm Hoptimase enzyme

1. GC-MS
2. Sensory
   - Are they different?
   - Scaling attributes
   - Descriptive analysis
3. Data

- GC
- MS
- GC/TOF

Data: Sensory, GC, GC/TOF

Cascade 250 g/hL

65 hours @ 15°C

1000 ppm Hoptimase enzyme

Are they different?
Scaling attributes
Descriptive analysis

Data
SPME-GCMS – IPA dry hopped with enzyme

- Isoamyl acetate loss in test = esterase side reactions (Daenen, 2012)
- Acetate esters hydrolyze more rapidly than ethyl esters – isoamyl acetate both chemically and enzymatically (Preedy, 2011)
Sensory – IPA dry hopped with enzyme

- If there is a difference, must be qualitative and outside scope of test
- Investigate further → descriptive analysis
Sensory – IPA dry hopped with enzyme

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual</td>
<td>Golden amber color with an orange glow, creamy white form and a heavy sheen</td>
<td>Same as control</td>
</tr>
<tr>
<td>Aroma</td>
<td>Mostly grapefruit and pine with some tropical pineapple, orange and caramel backin’ it up</td>
<td>Mostly pine with some citrus, orange and grapefruit, followed by tropical pineapple aroma as well, slight caramel and isoamyl acetate</td>
</tr>
<tr>
<td>Taste</td>
<td>Low sweetness into intense lingering bitter</td>
<td>Low to moderate sweetness with an intense bitter linger</td>
</tr>
<tr>
<td>Mouthfeel/Body</td>
<td>Medium body with a lingering astringency</td>
<td>Medium body, sl. creamy, finishes astringent</td>
</tr>
</tbody>
</table>

- Descriptive Analysis (n = 7 validated taste panelists)
- Overall most panelists noted that the **citrus, stone fruit and tropical aromas** were more pronounced in the test than the control
Next steps → Non-targeted GCO & TOF

- What are the aromas responsible for **citrus**, **stonefruit** and **tropical fruit** detected in sensory?
- GCO & TOF: What is different between test and control?
- Can we quantitate? Is it significant?
- Does this data correlate to sensory?
Polyfunctional thiols – hidden players?

- Primarily from fermentation by microbial metabolism of non-volatile precursors (Musumecci, 2015)
- Low sensory threshold, low concentrations, challenging to quantitate analytically
- Nelsen Sauvin—fruity volatile thiols (Gros, 2012)
- 3-sulfanyl-4-methylpentan-1-ol (3S4MP)
  - grapefruit, rhubarb aroma
  - May enhance flavor of terpene alcohols linalool & geraniol (Takoi, 2009)
In conclusion...

• Aroma active compounds are elusive – low concentrations may contribute to overall flavor due to synergistic and additive effect (Almaguer, 2014)

• Need sensory to validate analytical data

• Application and effectiveness of exogenous products may depend on process specifications

• Enzymatic reactions at dry hop warrant further investigation
Future directions

• Uses in process – dry hop? Enhance late/kettle hop additions?
• Enzyme action during fermentation (on yeast)
• Polyfunctional thiols – flavor enhancement potential
• Fruited and spiced beer
• Shelf life studies – flavor stability
• What glycosides are present in beer without hops?
• New method development to quantify compounds of interest
References


10. Kiyoshi Takoi, Koichiro Koie, Yutaka Itoha, Yuta Katayama, Masayuki Shimase, Yasuyuki Nakayama, and Junji Watari “Biotransformation of Hop-Derived Monoterpene Alcohols by Lager Yeast and Their Contribution to the Flavor of Hopped Beer” Journal of Agricultural and Food Chemistry 2010 58 (8), 5050-5058


Acknowledgements
- New Belgium Brewing Co. & the Quality Assurance Team
- DSM for the use of enzymes
- Our gracious hosts at Sierra Nevada

Thank you for your attention...
Questions?
Extras
Aroma precursors present in hops

• **Alcohols**: Aliphatic alcohols, terpene alcohols (sa. Linalool) (Kollmannsberger, 2006)
• B-damascenone glycosides in beer (Preedy, 2008)
• Increase in phenolic content when used in wine (Stepanova, 2006)

**Polyfunctional thiols**
• 41 thiols recently found in hop (Gros, 2012)
• Varietal specific cysteine-S-conjugate
• **Cascade** hops have high 3-sulphanylhexan-1-ol (grapefruit-like) potential (Gros, 2013)
Targeted analysis using SPME GC-MS

- Supelco 2 cm DVB/CAR/PDMS SPME fiber (10 minute extraction at 60°C)
- Gerstel MPS auto sampler (sample incubation 60°C x 5 min, agitation time of 60 sec)
- Agilent DB-5MS UI:2891.71150 60m x 320um x 1um
  - 40°C x 5 minutes
  - Ramp 4°C/min → 190°C
  - Ramp 30°C/min → 235°C x 1 min
- Agilent 7890A GC and 5975C MSD
- Compounds targeted via SIM:
  - Myrcene
  - Linalool
  - Caryophyllene
  - Humulene
  - Limonene
  - Geranyl acetate
  - 2-undecanone
Design of experiment (DOE)

• Two factors (enzyme and hopping rate)
• 7 responses and 5 repetitions
• Quantify changes using 7 hop aroma compounds with SPME GC-MS
  • Myrcene, limonene, linalool, 2-undecanone, geranyl acetate, caryophyllene, humulene

• GOAL:
  • Determine interactions between hop dosing rate and enzyme to optimize aroma compounds
  • Can access exogenous enzyme to enhance hop aroma and better utilization of dry hop load?
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<td>High geranyl acetate with high hopping rate, myrcene saturation?</td>
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<tr>
<td>Rapidase Expression Aroma (WG)</td>
<td>Aroma precursor extraction in white grape</td>
<td>Polygalacturonase and N-arabinofuranosidase</td>
<td>Hard to say what effects are (if any)</td>
</tr>
<tr>
<td>Rapidase Extra Fruit (RG)</td>
<td>Aroma precursor extraction in red grape</td>
<td>Polygalacturonase</td>
<td>High myrcene with high hopping rate, low geranyl acetate</td>
</tr>
</tbody>
</table>
DOE Results

• Alpha .05, but $R^2$ only 0.79 for linalool
• Challenge in measurement variation (SPME)
• Hopping rate = biggest driver
• Depends on enzyme (saturation and conditions)
• Red grape enzyme could be of interest (polygalacturonase)
• Enzyme effects likely to be unique per brand
TOF – IPA dry hopped with enzyme

- What is different? Can we quantitate? Is it significant?
- Does this data correlate to sensory?