Production of malic acid and poly(L-malic acid) from biomass hydrolysates by *Aureobasidium pullulans*

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The Ohio State University
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Introduction
Malic acid (MA)
- Food acidulant
- Additive in cosmetics
- Pharmaceuticals
- Potential building-block chemical
- Market: from 40,000 ton/yr to >200,000 ton/yr

\[
\text{HO} \quad \text{C} \quad \text{OH} \quad \text{HO} \\
\text{HO} \quad \text{C} \quad \text{O} \\
\]

Introduction
Introduction

Poly(L-malic acid) (PMA)

- Special Properties
  - Solubility in water
  - Biodegradability
  - Biocompatibility

- Potential Applications
  - Prodrug
  - Carrier in drug delivery systems: polycatin

<table>
<thead>
<tr>
<th>Biopolymers</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>hydrophilic, non-immunogenic, high encapsulation efficiency, lower release rate</td>
<td>possible fast hydrolysis at low molecular weight</td>
</tr>
<tr>
<td>Polylactic acid</td>
<td>high physical strength biodegradable</td>
<td>burst release (24hr) hydrophobic degrade product reduce pH</td>
</tr>
<tr>
<td>Polyglutamate</td>
<td>hydrophilic, biodegradable, edible</td>
<td>low thermostability, D-PGA has immunogenicity</td>
</tr>
</tbody>
</table>
**Introduction**

- **Aureobasidium pullulans**
  - Cosmopolitan yeast-like fungus
  - PMA synthesized from malic acid and secreted into fermentation broth
  - PMA recovered by ethanol precipitation or adsorption with anion-exchange resins and hydrolyzed into **malic acid**

**Production of MA and PMA**

![Chemical reaction diagram](image)

\[\text{Aureobasidium pullulans} \quad 0.88\text{g PMA} + 0.12\text{g H}_2\text{O} \rightarrow 1\text{g MA}\]
High level production of MA by *A. pullulans* ZX-10 from glucose has been achieved.

<table>
<thead>
<tr>
<th>Bioreactor</th>
<th>Mode</th>
<th>Time (hr)</th>
<th>MA Titer (g/L)</th>
<th>Yield (g/g)</th>
<th>Productivity (g/L*h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-L STR</td>
<td>Batch</td>
<td>96</td>
<td>47.3</td>
<td>0.47</td>
<td>0.49</td>
</tr>
<tr>
<td>5-L STR</td>
<td>Fed-Batch</td>
<td>140</td>
<td>87.6</td>
<td>0.49</td>
<td>0.61</td>
</tr>
<tr>
<td>FBB</td>
<td>Fed-Batch</td>
<td>192</td>
<td>142.2</td>
<td>0.55</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Investigation of low cost substrate for MA production
Selection of biomass feedstock
## Selection of biomass feedstock

**Lignocellulose/starch biomass**

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Initial sugar (g/L)</th>
<th>Malic acid (g/L)</th>
<th>Yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean Hull</td>
<td>48.04±0.21</td>
<td>36.80±1.70</td>
<td>0.77±0.039</td>
</tr>
<tr>
<td>Cotton stalk</td>
<td>59.82±0.70</td>
<td>34.45±0.35</td>
<td>0.58±0.013</td>
</tr>
<tr>
<td>Cassava bagasse</td>
<td>47.83±0.40</td>
<td>19.95±0.07</td>
<td>0.42±0.0050</td>
</tr>
</tbody>
</table>

- Pretreatment with enzyme.
- Soybean hull hydrolysate (SHH) gave the highest titer and yield.
- Soybean hull has an annual harvest of 7.8 million tons.
Selection of biomass feedstock

◆ Soybean molasses

• Composition of soybean molasses
  o 50% soluble solid

<table>
<thead>
<tr>
<th>Nitrogenous Substances</th>
<th>10%～20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minerals</td>
<td>10%</td>
</tr>
<tr>
<td>Fats</td>
<td>20%</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>40%～60%</td>
</tr>
</tbody>
</table>

• Advantage:
  o Other usage limited
  o Complex sugar composition
  o Easy pretreatment

Do not need ENZYME
Fermentation from soybean hull
Fermentation of soybean hull hydrolysate

◆ Pretreatment of soybean hull
◆ Shake flask results and optimization

<table>
<thead>
<tr>
<th>Initial sugar (g/L)</th>
<th>Malic acid (g/L)</th>
<th>Yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48.04±0.21</td>
<td>36.80±1.70</td>
<td>0.77±0.039</td>
</tr>
</tbody>
</table>

Optimized conditions:

- 80 g/L initial sugar (60 g/L, 80 g/L, 100 g/L, 120 g/L)
- 10 g/L corn steep liquor (NH₄NO₃, soybean meal, yeast extract, corn steep liquor)
Batch fermentation of soybean hull hydrolysate

Titer: 28.3g/L (60h), Yield: 0.40g/g, Productivity: 0.472g/L*h
Fed-batch fermentation of soybean hull hydrolysate

Titer: 31.3g/L (93.5h), Yield: 0.40g/g, Productivity: 0.335g/L*h
Repeated-batch fermentations with cell recycle

- **First batch**: fermentation using mixture of glucose and xylose.
- **Second batch**: cell recycle with 80% cells.
- **Third batch**: removal of 75% fermentation medium and add fresh medium of same volume

- **First batch**: Titer: 35.37 g/L (120 h), Yield: 0.701g/g, Productivity: 0.295 g/L*h
- **Second batch**: Titer: 27.04 g/L (44.5 h), Yield: 0.401g/g, Productivity: 0.481g/L*h
- **Third batch**: Titer: 28.06 g/L (47.25 h), Yield: 0.389g/g, Productivity: 0.499g/L*h
Fermentation from soybean molasses
Fermentation with soybean molasses

◆ Sugar composition of soybean molasses

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raffinose</td>
<td>20%</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20~40%</td>
</tr>
<tr>
<td>Glucose</td>
<td>0~20%</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0~20%</td>
</tr>
<tr>
<td>Stacchyose</td>
<td>10~30%</td>
</tr>
</tbody>
</table>

◆ Pretreatment of soybean molasses

H$_2$SO$_4$ pH→3.0, centrifuge; Ca(OH)$_2$ pH→6.0.

◆ Optimization

- 10 g/L corn steep liquor (no nitrogen source, NH$_4$NO$_3$, soybean meal, yeast extract, corn steep liquor, etc.)

Do not need ENZYME
Cell-recycle fermentation from soybean molasses

Titer of MA in the first three batches

<table>
<thead>
<tr>
<th>Batch</th>
<th>Raffinose</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Arabinose</th>
<th>Stacchyose</th>
<th>Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>11.27</td>
<td>25.39</td>
<td>0.61</td>
<td>2.16</td>
<td>17.43</td>
<td>0</td>
</tr>
<tr>
<td>After</td>
<td>0</td>
<td>4.09</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Yield of MA in the first three batches

- A. pullulans can consume all kinds of sugar in soybean molasses.
- Cell recycle can increase yield and titer.
Comparison with other work

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Nitrogen source</th>
<th>Initial sugar conc. (g/L)</th>
<th>Fermentation mode</th>
<th>Time (h)</th>
<th>MA titer (g/L)</th>
<th>Yield (g/g)</th>
<th>Productivity (g/L*h)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean hull</td>
<td>CSL</td>
<td>~70</td>
<td>Batch</td>
<td>60.3</td>
<td>28.3</td>
<td>0.4</td>
<td>0.47</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~70</td>
<td>Fed-batch</td>
<td>93.5</td>
<td>31.3</td>
<td>0.4</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>~60</td>
<td>Cell recycle</td>
<td>40.5</td>
<td>27.0</td>
<td>0.4</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>~50</td>
<td>Repeated batch</td>
<td>37</td>
<td>28.1</td>
<td>0.39</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Soybean molasses</td>
<td>CSL</td>
<td>~70</td>
<td>Cell recycle</td>
<td>-</td>
<td>41.6</td>
<td>0.69</td>
<td>-</td>
<td>Zan et al, 2013</td>
</tr>
<tr>
<td>Sweet potato</td>
<td>Yeast extract</td>
<td>120</td>
<td>Batch</td>
<td>120</td>
<td>34</td>
<td>0.32</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fed-batch</td>
<td>156</td>
<td>50</td>
<td>0.22</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fed-batch FBB</td>
<td>156</td>
<td>65.3</td>
<td>0.23</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>Yeast extract</td>
<td>50</td>
<td>Batch</td>
<td>168</td>
<td>26.7</td>
<td>0.53</td>
<td>0.16</td>
<td>Leathers et al, 2013</td>
</tr>
</tbody>
</table>


Conclusions

◆ Pros

• Soybean hull and soybean molasses are promising feedstock for fermentative production of MA and PMA by *Aureobasidium pullulans*

• Cell recycle is effective in improving fermentation productivity from soybean hull, and in improving yield and titer from soybean molasses

◆ Cons

• Inhibition of growth at high concentration (e.g. Soybean hull hydrolysate with total sugar \( \geq 120 \text{ g/L} \))
Future work

- Possible way to improve PMA fermentation from soybean hull hydrolysate
  - Pretreatment of soybean hull hydrolysate for detoxification
  - Study the effects of concentration of nitrogen source

- Possible way to improve PMA fermentation from soybean molasses
  - High concentration of soybean molasses
  - Detoxification of soybean molasses

- Other kinds of biomass
Acknowledgements

◆ United Soybean Board – financial support
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Thank you!