Developing novel strategies on laccase production and purification: Recent advances and new trends

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Bio 2013, San Diego
Laccase

- Belonging to a group of blue multicopper oxidases
- Catalyzing one-electron oxidation coupled to the reduction of molecular oxygen to water

*Rhus vernicifera*  
*Trametes versicolor*  
*Bacillus subtilis*
Application of laccase

- Environmental bioremediation: Pollution degradation
- Dyeing and textile industry: Dye decolorization
- Paper and pulp processing: Pulp delignification and bleaching
- Food industry: Removal of phenolic compounds
- Analyte detection: Biosensor
- Organic synthesis: Biopolymer modification

Purified enzyme
Barrier for industrial applications of laccase

Problems

Long culture period
Low laccase yield

High cost

Difficult for laccase purification

Strategies

✓ Isolation and breeding of high producing strains
✓ Optimization of culture medium
✓ Heterologous expression of laccase genes

✓ Chromatography technology
✓ Foam fractionation
✓ Three-phase partitioning
Ultrasound-intensified laccase production

from *Trametes versicolor*
Ultrasound-intensified laccase production

Culture period: 144 h
Laccase activity: 328.9 U/L

Time course of laccase production by *T. versicolor*
Effect of the ultrasonic treatment on laccase production at different cultivation stages

- Control
- US D1
- US D2 443.8 U/L
- US D3
- US D4
- US D5 450.7 U/L
- US D6
Effect of the duration of ultrasonic treatment on laccase production

(Optimal ultrasonic duration: 5 min)
Effect of the multiple ultrasonic treatment on laccase production.

(A and B) After 2 days culture;

(C) After 5 days culture

US 2 times, 12 h interval after 2 days culture: 588.9 U/L

Effect of the multiple ultrasonic treatment on laccase production.

(A and B) After 2 days culture;

(C) After 5 days culture
Ultrasound-intensified laccase production

Culture period: 120 h
Laccase activity: 588.9 U/L
79.1% greater than control

Time course of laccase production stimulated using the optimized ultrasonic treatment strategy
Possible mechanism of ultrasonic stimulation

Effect of the ultrasonic treatment on the pellet porosity using the optimized ultrasonic treatment strategy
Possible mechanism of ultrasonic stimulation

Laccase activity released from pelleted mycelia during the ultrasonic treatment
Possible mechanism of ultrasonic stimulation

Stimulation of lcc expression using the optimal ultrasonic strategy.

- a&c, lcc expression at different cultivation stages;
- b&d, actin gene expression at different cultivation stages;
- e&g, lcc expression following ultrasonic treatment;
- f&h, actin gene expression following ultrasonic treatment.
Possible mechanism of ultrasonic stimulation

Effect of the ultrasonic treatment on intracellular (ILA) and extracellular (ELA) laccase activity using the optimized ultrasonic treatment strategy
Application of ultrasonic strategy in laccase production with immobilized cells

Time course of laccase production by immobilized *T. versicolor*

Culture period: 72 h
Laccase activity: 503.3 U/L
Application of ultrasonic strategy in laccase production with immobilized cells

Time course of laccase production stimulated using the optimized ultrasonic strategy for immobilized *T. versicolor*

Culture period: 72 h
Laccase activity: 1087.4 U/L
116% greater than control

Time (h)
Laccase activity (U/L)
Reducing sugar (g/L)
Protein (mg/mL)
Application of ultrasonic strategy in laccase production with immobilized cells

Schematic diagram of the bubble column reactor
Application of ultrasonic strategy in laccase production with immobilized cells

Laccase production by immobilized *T. versicolor* with the optimized ultrasonic strategy in the bubble column reactor
Functionalized magnetic mesoporous silica nanoparticles: fabrication, laccase adsorption performance and direct laccase capture from *Trametes versicolor* fermentation broth
Immobilized metal ion affinity chromatography (IMAC)
Size selectivity of mesopore for protein adsorption

(1) Fabrication of magnetic mesoporous silica nanoparticles

\[ \text{Fe}_3\text{O}_4 \rightarrow \text{Diamine surfactant} \rightarrow \text{TEOS} \rightarrow \text{Calcine} \]

(2) Surface modification

\[ \text{CPTS} + \text{CH}_3\text{OH} + \text{H}_2\text{O} \rightarrow \text{Si} - \text{Cl} \rightarrow \text{IDA} \]

\[ \text{Si} - \text{N} \rightarrow \text{Cu} \rightarrow \text{OH}_2 \rightarrow \text{OH}_2 \rightarrow \text{OH}_2 \]
Characterization of MMSNPs

Disordered mesopore

X-ray diffraction patterns

N$_2$ adsorption-desorption isotherm
Characterization of MMSNPs

TEM images

25°C

45°C

Wormhole mesostructure

60°C

80°C
Characterization of MMSNPs

Pore size distribution curve

- 25 °C  3.6 nm
- 45 °C  7.0 nm
- 60 °C  14.5 nm
- 80 °C  27.1 nm

Pore size (nm)

Pore size distribution curve
### Characterization of MMSNPs

#### Properties of MMSNPs synthesized at different temperatures

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>$S_\text{BET}$ (m$^2$/g)</th>
<th>BJH pore size (nm)</th>
<th>Pore volume (cm$^3$/g)</th>
<th>Particles size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>820</td>
<td>3.6</td>
<td>0.82</td>
<td>485</td>
</tr>
<tr>
<td>45</td>
<td>735</td>
<td>7.0</td>
<td>1.43</td>
<td>395</td>
</tr>
<tr>
<td>60</td>
<td>579</td>
<td>14.5</td>
<td>2.14</td>
<td>287</td>
</tr>
<tr>
<td>80</td>
<td>501</td>
<td>27.1</td>
<td>3.00</td>
<td>213</td>
</tr>
</tbody>
</table>
Laccase adsorption and desorption in a model system

Effect of pH on laccase adsorption onto MMSNPs-Cu$_2^+$

Laccase pI ~ 3.5
**Dynamic adsorption curve of laccase onto MMSNPs-Cu$^{2+}$**

**Equilibrium adsorption isotherm for laccase onto MMSNPs-Cu$^{2+}$**
Direct laccase capture from fermentation broth

Lane 1: marker, Lane 2: broth, Lane 3: laccase standard (sigma), Lane 4-7: purified laccase by surface modified MMSNPs synthesized at 25 °C, 45 °C, 60 °C, 80 °C, respectively.

Reducing SDS-PAGE of purified laccase from *T. versicolor* culture supernatant

MW: ~65 kDa
### Direct laccase capture from fermentation broth

<table>
<thead>
<tr>
<th>Magnetic supports</th>
<th>Recovered protein (ug/g supports)</th>
<th>Recovered activity (U/g supports)</th>
<th>Specific activity (U/mg)</th>
<th>Fold purification</th>
<th>Activity yield (%)</th>
<th>Particles dosage (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMSNPs-Cu²⁺-25</td>
<td>176.9</td>
<td>3.1</td>
<td>17.5</td>
<td>3.9</td>
<td>47.2</td>
<td>2.50</td>
</tr>
<tr>
<td>MMSNPs-Cu²⁺-45</td>
<td>326.6</td>
<td>86.0</td>
<td>263.3</td>
<td>58.5</td>
<td>78.7</td>
<td>0.15</td>
</tr>
<tr>
<td>MMSNPs-Cu²⁺-60</td>
<td>690.0</td>
<td>188.0</td>
<td>272.5</td>
<td>60.6</td>
<td>114.6</td>
<td>0.10</td>
</tr>
<tr>
<td>MMSNPs-Cu²⁺-80</td>
<td>776.0</td>
<td>19.6</td>
<td>25.3</td>
<td>5.6</td>
<td>59.8</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Note: All the laccase in the broth was adsorbed onto the supports in each experiment; culture supernatant: 50 mL, 3650 ug of total protein, 16.4 U of total activity.
Repeated use of MMSNPs-Cu$^{2+}$-60 for laccase purification from the culture supernatant.
**Direct laccase capture from fermentation broth in MSFB**

Laccase purification in magnetically stabilized fluidized bed (MSFB)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (mg)</th>
<th>Laccase activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification folds</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>115.3</td>
<td>519.6</td>
<td>4.5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Elution</td>
<td>2.014</td>
<td>565.7</td>
<td>280.9</td>
<td>62.4</td>
<td>108.9</td>
</tr>
</tbody>
</table>

[Diagram of laccase purification setup]

[Graph showing protein concentration and laccase activity vs. volume]
Conclusion

- An efficient ultrasonic treatment strategy was developed to improve laccase production from free and immobilized *Trametes versicolor*.

- Increase in laccase production is related to the increase in pellet porosity, the release of intracellular laccase into the extracellular culture media, and the enhanced gene expression of the laccase biosynthesis.

- Purification using the MMSNPs-Cu$^{2+}$ with suitable pore size resulted in laccase isolated with a high activity yield and to a purity level comparable to standard laccase.

- This simple and efficient strategy has the potential to be used for the robust and inexpensive preparation of purified laccase in MSFB.
Thank you!