TEKNOLOGI BIOETANOL BERBASIS LIMBAH LIGNOSELULOSA

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Pusat Penelitian dan Pengembangan Keteknikan Kehutanan dan Pengolahan Hasil Hutan
Kementrian Lingkungan Hidup dan Kehutanan
Bogor

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• Chapter 1. Pendahuluan
• Chapter 2. Amphipathic lignin derivatives dari lindi hitam sebagai limbah proses pulping
• Chapter 3. Hidrolisis enzimatis pulp sagu dengan penambahan SSL-derivatives sebagai surfaktan
• Chapter 4. Penutup
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Chapter 1.
General introduction

High level pollution

Petroleum, coal, natural gas, etc

Developing biomass as an alternative feedstock to biofuels:
• Abundant, produced about 10-50 billion tons/year
• Renewable
• Bioethanol

1\textsuperscript{st} generation starch

\rightarrow

2\textsuperscript{nd} generation lignocellulose

Agricultural and forestry residues (waste)

• Simple process to produce bioethanol
• But, \textit{compete with production of daily food.}

raising the price of the food feedstock

\rightarrow

• Never compete with food production.
• Produced from all kinds of plant.

Effective utilization of unused lignocellulose

• Sago palm

Sago palm

\rightarrow

Fibrous sago waste from sago mills

Promising lignocellulosic for bioethanol production
**Bioethanol of lignocellulose**

1. Acid hydrolysis
   - **Advantages**: Fast process, Cheap reagent
   - **Disadvantages**: Corrosive, High cost of maintenance

2. Enzymatic hydrolysis
   - **Advantage**: Mild condition, Environmentally friendly
   - **Disadvantages**: High cost of enzyme, Pretreatment

Lignocellulose → Glucose → Bioethanol

**Enzymatic hydrolysis or saccharification**

Without delignification, enzymatic saccharification is very low (< 20%)
• Delignification for substrates

Raw materials lignocellulosic

Pretreatment

- Physical (crushing or milling)
- Biological (white-rot fungi)
- Chemical (alkaline, acid)
- Combined method (steam explosion + alkaline)

Alkaline pulping

Delignified pulp

Enzymatic saccharification

• What is the enzyme used for saccharification?

Cellobiohydrolase (CBH)
...hydrolyze cellulose to cellobiose

Endoglucanase (EG)
...hydrolyze randomly internal glycosidic bonds

β – Glucosidase
...hydrolyze cellobiose to glucose

- CBH I (Cel7A)
  Mw : 59-68 kDa  pI : 3.5-4.2
- CBH II (Cel6A)
  Mw : 50-58 kDa  pI : 5.1-6.3
- EG I (Cel7B)
  Mw : 50-55 kDa  pI : 4.0-6.0
- EG II (Cel5A)
  Mw : 48 kDa  pI : 5.5
- BGL (Cel3A)
  Mw : 75 kDa  pI : 8.7
• Why cellulase activity decreased during saccharification?

One of obstacles in enzymatic saccharification is irreversible adsorption of cellulase to cellulose

• Immobilized cellulase

Water-soluble immobilized enzyme

Amphiphilic lignin derivative

E: Enzyme, S: Substrate, P: Product
• Positive effect of surfactant as a support of water immobilized enzyme


2. SSF (Simultaneous saccharification and Fermentation). (Alkasrawi et al, 2003)

3. Re-use of cellulase is possible. (Otter et al, 1989; Tu et al, 2007)

Developed amphipathic lignin derivatives as alternative surfactant from lignin

• Hypothesis on the mechanism of cellulase saccharification using lignin derivatives

It is expected that lignin derivatives promotes the release of cellulase from substrate.

With lignin derivatives
Possible to repeat a cellulase hydrolysis
**Repeated cellulase saccharification**


Hydrolysis conditions: Substrate, 3 g; Cellulase, 240 mg; PEGDE-AL, 0 wt.% (●) and 0.2 wt.% (●) based on 300 mL of buffer solution

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**Objectives of the research**

1) To synthesize amphipathic lignin derivatives from sago lignin

2) To evaluate the effect on the sago lignin derivatives to the saccharification of sago pulp

3) To examine the possibility of re-use of cellulase that recovered from hydrolysate of sago pulp saccharification
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Introduction

Sago (Metroxylon sp.)

One of Indonesian native plants Food and energy

Starch from sago plants used as various types of dishes or as main daily food in Maluku and Papua provinces.
**Introduction**

Sago potency: 1.25 million ha

- (Mentawai) 56,000 ha
- (Riau) plantation 20,000 ha
- (Pariaman) 95,790 ha
- (Borneo) 2,795 ha
- (Sulawesi) plantation 30,000 ha
- (Irian Jaya) plantation 1,206,469 ha
- (Maluku) 50,000 ha
- (Sulawesi) plantation 30,000 ha
- (Borneo) 2,795 ha

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1. **Fibrous sago waste**
   - Pretreatment (4% HCl; 80°C; 1 h)
   - Starch-free sago waste (Yield = 51.2%)

2. **Soda pulping**
   - (wood to liquor ratio = 1 : 5); 165°C (heating time: 2 h; holding time: 1.5 h)

3. **Black liquor**
   - (adjust to pH 4; filtration freeze-drying)

4. **Sago soda pulp (substrate)**
   - (Yield = 30-40%)
   - NaOH 15% NaOH 20%
   - KL: 10.2% KL: 1.8%

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- **Enzymatic hydrolysis** (using Cellulase, 10 FPU/g substrate; 48 h)
- **Amphipathic lignin derivatives**
- **Epoxylated PEGs**
- **Sago soda Lignin (SSL)**
- **Ultrafiltration**
- **Reuse**
- **glucose**
1. Preparation of sago soda pulp

Fibrous sago waste (9.7% starch); 4% HCl (aq) to remove and recovered the starch

Alkaline pulping

Filtration

Centrifugation

Dry pulp

Unbleached Sago pulp

Starch free sago waste

2. Enzymatic hydrolysis

Soda pulping (wood to liquor ratio = 1:5; 165°C; heating time: 2h; holding time: 1.5 h)

(adjust to pH 4; filtration freeze-drying)

Epoxylated PEGs

Amphipathic lignin derivatives

Sago soda Lignin (SSL)

Black liquor

Fibrous sago waste

Pretreatment (4% HCl; 80°C; 1 h)

Starch-free sago waste

(Yield = 51.2%)

Sago soda pulp (substrate)

(Yield = 30-40%)

Reuse

Enzymatic hydrolysis (GC220, 10 FPU/g substrate; 48 h)

Ultrafiltration
2. Preparation of sago soda lignin (SSL)

Fig. Separation scheme of sago soda lignin

Polyethylene glycol derivatives

1) **PEGDE** Polyethylene glycol diglycidylether

![Chemical structure of PEGDE]

2) **EPEG** ethoxy-(2-hydroxy)-propoxy-polyethylene glycol glycidylether

![Chemical structure of EPEG]

3) **DAEO** dodecyloxy-polyethylene glycol glycidylether

![Chemical structure of DAEO]

2. Preparation of (PEGDE-; EPEG-; DAEO-) SSL

SSL and 1 M NaOH

Stirring 24 h

PEGDE, EPEG, DAEO added to the solution

Stirring at 70°C for 2 hr

Checking the solubility in water

1) pH 4 with AcOH
2) Ultrafiltration
3) Lyophilization

Amphipathic SSL derivatives (PEGDE-; EPEG-; DAEO-SSL)
Dissolve in water or amphipathic

- Characterization of lignin derivatives-SSL

PEGDE-SSL (13, 1)
EPEG-SSL (13, 1.5)
DAEO-SSL (3) \((n,m)\)

\(n\): the number of EO unit
\(m\): charge ratio of PEG (g) to 1 g of SSL

3. Measurement of surface activity

Surface tension

Water (at 20°C): 72.8 mN/m
aqueous solution of commercial surfactant: 30~40 mN/m

Du Nouy surface tensiometer

Surface tension

Log concentration

Intersection point of 2 approximate line

\(...\)critical micelle concentration (CMC)
The lower CMC, the better surfactant
Homma et al, 2010; J. Wood Chem. Technol. 30 : 164-174

PEGDE-AL(13,1)  
Surface tension (mN/m)  
Concentration (g/mL)  
CMC: 6.3 x 10^3 g/mL  
Surface tension: 45 mN/m

EPEG-AL(13,4)  
Surface tension (mN/m)  
Concentration (g/mL)  
CMC: 5.0 x 10^3 g/mL  
Surface tension: 36 mN/m

DAEO-AL(6)  
Surface tension (mN/m)  
Concentration (g/mL)  
CMC: 2.0 x 10^4 g/mL  
Surface tension: 34 mN/m

Lower critical micelle concentration (CMC) and lower surface tension indicate higher surface activity.

PEGDE-AL < EPEG-AL < DAEO-AL

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3. Saccharification of unbleached sago pulp

- Measurement of Saccharification efficiency

\[
\text{Saccharification efficiency} \% = \left( \frac{W_S - W_R}{W_S} \right) \times 100
\]

- Washed and reuse until 4 times, check the residual activity using FPU method

Buffer solution: 50 mL (pH 4.8 citrate buffer)

amphipathic lignin derivative (PEGDE-SSL, EPEG-SSL, DAEO-SSL): 0.05 g

Cellulase (Genencor GC 220): 10 FPU/g of pulp

Substrate (unbleached sago pulp): 0.5 g (1.8% and 10.2% lignin content)
- **Saccharification efficiency of sago pulp**

  Lignin content 1.8%

  ![Graph showing saccharification efficiency](image)

  - All lignin derivatives showed higher efficiency than without additive.
  - DAEO-SSL showed the highest SE among the others.

- **Saccharification efficiency of sago pulp**

  Lignin content 10.2%

  ![Graph showing saccharification efficiency](image)

  - All lignin derivatives showed higher SE than without lignin derivatives
  - PEGDE-SSL showed the highest efficiency among others
• Residual cellulase activity after 4 times saccharification

DAEO-SSL showed the highest residual cellulase activity after 4 times saccharification for unbleached sago pulp (1.8% lignin content).

EPEG-SSL showed the highest residual cellulase activity after 4 times saccharification of unbleached sago pulp (10.2% lignin content).

Chapter 4. Penutup

• SE of unbleached sago pulp was improved by the addition of amphipathic lignin derivatives, particularly DAEO-SSL.

• The recovered cellulase in the presence of the lignin derivatives was able to be used again and maintained until 4 times saccharification.

• DAEO-SSL showed a superior performance to others derivatives; can maintain cellulase activity particularly for pulp with lignin content of 1.8%.

It is possible to re-use cellulase for enzymatic saccharification of unbleached sago pulp.
Acknowledgement

1. Prof. Dr. Uraki Yasumitsu
2. Dr. Keichii Koda
3. All my lab mates during my doctoral course
• Saccharification efficiency without lignin derivatives of Meicelase and GC220 and cellulase content

• Different types of commercial cellulases (no additives)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Meicelase (unit/ FPU)</th>
<th>GC 220 (unit/ FPU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC (EG)</td>
<td>67.0</td>
<td>162.8</td>
</tr>
<tr>
<td>$\rho$-nitrophenyl lactoside (CBH)</td>
<td>1.4</td>
<td>3.5</td>
</tr>
<tr>
<td>$\rho$-nitrophenyl glucoside ($\beta$-glucosidase)</td>
<td>8.2</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Genencor GC220 showed a little superior performance to Meicelase
• AL-derivatives maintained the residual cellulase activity at a higher level than PEG4000 and without additive after one-time saccharification.

• Amphipathic AL derivatives showed directly associated with CBH II, whereas PEG4000 did not.

• DAEO-SSL showed a superior performance to others derivatives for improvement SE and maintained cellulase activity until 4 times saccharification.

• Therefore, I confirmed that amphipathic lignin derivatives are very good materials as a cellulase aid-agent, because they can maintain and recover the cellulase activity.

This research will contribute to provide a useful method for bioethanol feedstock with low production cost from 2nd generation, in particular utilization of cedar wood from forest thinning and sago waste from sago mills.


**ACTIVITIES**

**Paper**


**Conference**

1. Ina Winarni; Chihiro Oikawa; Keiichi Koda; Yasumitsu Uraki; Tatsuhiko Yamada: Improvement of cedar pulp with amphiphilic lignin derivatives, 62rd Annual meeting of Japan Wood Research Society. Sapporo, March 2012.

2. Ina Winarni; Chihiro Oikawa; Tatsuhiko Yamada; Keiichi Koda; Yasumitsu Uraki: Improvement of cedar (Cryptomeria japonica) pulp with amphiphilic lignin derivatives as a cellulase aid-agent, International Conference of International Union of Forest Research Organization; Division 5, Lisbon-Portugal, July 2012.

3. Ina Winarni; Chihiro Oikawa; Tatsuhiko Yamada; Keiichi Koda; Yasumitsu Uraki: Amphiphilic lignin derivatives to improve enzymatic saccharification of lignocelluloses, CABS, Sapporo, August, 2012.
• Interaction of amphipathic lignin derivatives and PEG4000 with CBH II

Measuring instrument: Biacore

Detect physical interactions between molecular (amount of adsorption and desorption)

![Diagram of Biacore instrument showing light source, detector, sensorchip, and flow direction]
Repeating hydrolysis of filter paper (A) and PHA-pulp(B) By cellulase and PE-AL – cellulase complex

Hydrolysis conditions: Substrate, 3 g; Cellulase, 240 mg; PE-AL, 0 wt.% (●) and 0.2 wt.% (○) based on 300 mL of buffer solution


How does PE-AL effect on cellulase?

- remained epoxy group would reacts with other functional groups on cellulase to yield covalent bond.
• Why the complete recovery can be achieved?

The amount of enzyme required to liberate 1 μmol of glucose from filter paper/minute for 1 h

NREL/TP-510-42628 (Ghose, 1987)

Meicelase

Limiting step

To produce cellobiose

(Cellobiose to glucose)

-less endoglucanase

GC220

Easy to prepare cellobiose

(Cellobiose to glucose)

(much endoglucanase)

Sensorgram the interaction between CBH II with amphipathic lignin derivatives and PEG4000
Amphipathic lignin derivatives

Repeated hydrolysis of and PHA-pulp by cellulase and PEGDE-AL-cellulase complex

PEGDE-AL is an amphipathic lignin derivative prepared from acetic acid lignin(AL) and polyethylene glycol derivative.

PE

EPEG, DAEO

unstable

stable

EPEG-AL and DAEO-AL are more stable than PE-AL

Homma et al, 2010; J. Wood Chem. Technol. 30 : 164-174

PEGDE-AL(13,1)

CMC: 6.3 x 10^{-3} g/mL
Surface tension: 45 mN/m

EPEG-AL(13,4)

CMC: 5.0 x 10^{-4} g/mL
Surface tension: 36 mN/m

DAEO-AL(6)

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- Lower critical micelle concentration (CMC) and lower surface tension indicate higher surface activity.

PEGDE-AL < EPEG-AL < DAEO-AL
The general understanding of cellulose degradation at present is that for efficient breakdown of crystalline cellulose, three types of enzymes are needed. They are endo glucanases (EGs, which cut cellulose chains randomly), cellobiohydrolases (CBHs, which cleave cellobiose from the cellulose chain ends) and b-glucosidases (which hydrolyze cellobiose and cellodextrins into glucose). Efficient cellulose degradation by microbes is achieved in two major ways. Production of a ‘soup’ of different enzymes that can work synergistically is one method. These different enzymes could have differential expression depending on the substrate, time and other stimuli. The second method is cellulosome activity. In this approach, different enzymes are found associated as one complex (the cellulosome) for fast breakdown of cellulose.
Meicelase

Enzyme adsorption to substrate

In particular endoglucanase occurred at 10 FPU

Reduction of cellobiose production

Reduction in FPU

GC 220 (Genencor)

Enzyme adsorption to substrate

Still remained endoglucanase activity (much production of cellobiose)

No apparent reduction of FPU