Enzymatic Saccharification and Ethanol Production of Xylem from Purwodadi Botanical Garden Trees

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Abstract

Recent studies have revealed that sengon (*Paraserianthes falcataria*) xylem consists of soft walls which are easily hydrolysable with a commercial cellulase preparation. We felt it important to determine the saccharification levels for fast-growing tropical trees, of which sengon, one of the fastest growing tree species in Indonesia, was used as the control wood species. The aim of this study was to screen and evaluate the xylem of Purwodadi Botanical Garden trees for saccharification in order to assess their potential usefulness in bioethanol production. Saccharification and fermentation were first examined in the xylem derived from the branches of 32 trees. The xylem was then milled into powder using a ball mill, and the powdered xylem was digested with a commercial cellulase preparation (Accelerase, Palo Alto, USA) derived from *Trichoderma viride.* The levels of enzymatic hydrolysis of cellulose and ethanol production were higher for *Firmiana malayana* and *Pterocarpus indicus* than for sengon.

Keywords: saccharification and fermentation, xylem, enzymatic hydrolysis, ethanol production.

Introduction

Raw materials for bioethanol production are still dominated by food plants (generation I), such as corn, sugar-cane, starch, etc. This will invariably produce a conflict of interest due to competition with food supply needs, resulting in increased food prices. This situation will only worsen as it has been predicted that environmental issues such as global warming will threaten the global food supply. Furthermore, the availability of these raw materials is not guaranteed. In general, even the current total global production of starch and sucrose (1.5×10^9 tonnes/year) that can be converted into bioethanol can only satisfy at most 8×10^{11} L/year while the demand for world fuel currently stands at 1.2×10^{12} L/year (Hayashi 2009). Therefore, alternative sources of raw materials other than food crops are needed, of which the non-food lignocellulosic materials (generation II) are promising candidates.

Wood-derived lignocellulosic biomass is a good alternative raw material for bioethanol that would benefit from further development. Wood offers several advantages, such as a high cellulosic content (≥80% holocellulose). In addition to its benefits, wood can be planted in marginal land, unlike agriculture crops (Kaida *et al*. 2009). The mixing of 85% wood-based bioethanol (E85) with fossil fuel can reduce carbon emissions by 65%, while the mixing of starch-based bioethanol with fossil fuel can reduce emissions by only 17~23% (Watanabe 2008). Additionally, raw bioethanol material from wood does not require a large storage space, unlike other lignocellulosic materials. Wood can also be cultivated in an Industrial Forest Estate (IFE) to further ensure a continuous supply. This industrial forest can produce up to 5.0×10^9 tonnes/year of cellulose that can be converted into 2.6×10^{12} L of bioethanol (Hayashi 2009). Wood plantation in an IFE area is compatible with the Kyoto Protocol since these planted trees can increase the carbon stock on earth, which will ultimately reduce green-house gasses in the atmosphere.

Plant-derived fuel is a potential alternative energy source because of the abundant production of biomass in Indonesia. Therefore, the continuity of plant-derived fuel is more probable than that of fossil fuel. Indonesia has various wood species that, if properly analyzed, will strengthen its position as a leading producer of bioethanol in the future.

The main problem of bioethanol production from lignocellulosic biomass, especially wood, is due to the characteristics of plant cell walls which make it difficult to achieve enzymatic hydrolysis. This drawback limits the utilization of this biomass and makes ethanol production from cellulose uneconomical. Hayashi (2009) mentioned that wood is highly resistant to enzymatic degradation, which inhibits its degradation into fermented sugar. Therefore, easy accessibility of both saccharification and fermentation are considered the most important factors in the conversion of wood to bioethanol.

Research into a simplified hydrolysis process and the conversion of a wood species into bioethanol is still uncommon enough that there is considerable opportunity to investigate certain wood species. Recent studies revealed that sengon xylem consists of soft walls which are easily hydrolysable with commercial cellulase preparations. On the other hand, mangium xylem consists of hard walls which are less hydrolysable than those of sengon, although the lignin content is lower for mangium than for sengon. Genetically loosened walls increased the level of saccharification from 30% to 60% in the case of sengon and from 10% to 15% in the case of mangium (Kaida *et al*. 2009). We believed it important to evaluate the saccharification levels of fast-

growing tropical trees, of which sengon and mangium are the fastest growing in Indonesia. Enzymic saccharification was employed in this study because the hydrolysis employed is much more environmentally friendly than are other processes such as acid hydrolysis.

Many valuable species of trees have been found in the Indonesian forests. In addition, many tropical trees have been brought to Indonesian botanical gardens from all over the world since the Dutch era. Purwodadi Botanical Garden is located at Pasuruan, 70 km from Surabaya, East-Java, at an elevation of 400 m above sea level. The area covers 85 Ha and contains 738 woody plants (Soewilo *et al*. 1999). The aim of this study was to screen and assess the xylem of 32 Purwodadi Botanical Garden trees for saccharification in order to assess their potential usefulness in bioethanol production.

Materials and Methods

Xylem Preparation

Single branches at a height of 2 to 3 m above the ground were removed from 32 trees located in Purwodadi Botanical Garden, Indonesia. The bark was peeled away and the xylem of each branch was dried in an oven at 70°C. The xylem was then milled to a powder using a ball mill at a speed of 15 rps for 30 min. The powder was used as a xylem preparation for saccharification alone or in combination with fermentation.

Wall Analysis

The xylem preparation was ground in liquid nitrogen, and the resulting fine powder was successively extracted four times with water and 24% KOH containing 0.1% $NaBH_4$. The insoluble wall residue (cellulose fraction) was washed twice with water. The amount of cellulose was determined by measuring the acid-insoluble residue; the samples were extracted with an acetic/nitric reagent (80% acetic acid/concentrated nitric acid, 10:1) in a boiling water bath for 30 min (Updegraff 1969) and the resulting insoluble material was washed in water and freeze-dried. The total sugar in each fraction was determined by the phenol-sulfuric acid method (Dubois *et al.* 1956). The lignin content was determined by the Klason method (Chiang and Funaoka 1990).

Enzymatic Hydrolysis

Precisely 50 mg of each xylem preparation was impregnated with water, autoclaved at 120°C for 3 min, and washed once with water by centrifugation. A commercial cellulase preparation (Accelerase, Palo Alto, USA) derived from *Trichoderma viride* was used to digest the xylem. The enzyme preparation contained endocellulases, exocellulases (CBHI and CBHII), xyloglucanase, xylanase, galactanase, and polygalacturonase. Enzymatic hydrolysis of the xylem preparation was performed in 1 ml of 50 mM sodium acetate buffer, pH 4.8, containing 0.02% Tween 20 and 0.4 filter paper units of a cellulase preparation (2.0 mg). One filter paper unit was defined as 1 µg of glucose released/minute from the filter paper. The mixture was incubated at 45°C in a rotary shaker set at 75 rpm. Approximately 100 µl of the supernatant was collected at 6, 12, 24, and 48 h of hydrolysis and used for sugar analysis. The sugar released was estimated as reducing sugar by the Nelson-Somogyi method (Somogyi 1952). Furthermore, free sugars released were directly analyzed according to their alditol acetates using gas chromatography (Hayashi 1989).

Ethanol Production

Mixtures were produced, each of which contained one type of xylem preparation in 1 ml of 50 mM sodium acetate buffer, pH 4.8, 0.02 % Tween, 0.4 filter paper units of a cellulase preparation and a seed culture of *Saccharomyces cervisiae* (SH1089) with yeast nutrients (4 mg $(NH_4)_2HPO_4$, 0.2 mg $MgSO_4/7H_2O$, and 8 mg yeast extract). Each mixture was incubated at 37°C in a rotary shaker set at 100 rpm and then subjected to simultaneous enzymatic saccharification and fermentation. About 100 µl of the supernatant was collected at 48 h of hydrolysis and used for ethanol analysis. The ethanol formed was measured by gas chromatography on a Supelcowax-10 column (0.53 mm i.d. × 15 m; Supelco, Bellefonte, PA, USA) at 50°C using an Agilent gas chromatograph. Butanol was used as an internal standard.

Results and Discussion

Enzymatic Saccharification and Ethanol Production

The levels of enzymatic saccharification varied among the xylem samples from the 32 trees. At 48 h, the highest level of saccharification was obtained from the xylem of *Firmiana malayana*, which had released 36.9 mg of sugars/100 mg xylem. In contrast, sengon released 29 mg of sugars/100 mg xylem (Kaida *et al*. 2009). A higher level of cellulose hydrolysis was also observed in *Pterocarpus indicus* (34.0 mg) than in sengon.

Composition of Xylem

The results showed that the cellulose amounts ranged from 8.0% (*Alstonia scholaris*) to 54.8% (*Acacia catechu*). Hemicelluloses ranged from 5.6% (*Lagerstroemia speciosa*) to 26.9% (*Pterocymbium javanicum*). Lignin contents ranged from 23.3% (*A. catechu*) to 36.9% (*Syzygium polyanthum*).

A. catechu had the highest cellulose content, yet its saccharification level was only 28.4 mg/100 mg xylem. Therefore, the levels of enzymatic saccharification were not directly related to the cellulose content. *L. speciosa* had the lowest hemicellulose content, yet it released only 12.0 mg of sugars/100 mg xylem. Therefore, the levels of enzymatic saccharification were not related to the hemicellulose content. Although xyloglucanase activity improved the total hydrolysis of lignocelluloses (Benko *et al*. 2008), there was

no correlation between ethanol production and xyloglucan content. Lignin is known to be a recalcitrant compound in cellulose hydrolysis (Chen and Dixon 2007). The data showed a correlation between high enzymatic saccharification and low lignin content, but there were exceptions. *A. catechu* had the lowest lignin content, yet it released only 28.4 mg of sugars/100 mg xylem. Therefore, the levels of enzymatic saccharification were also not related to the lignin content.

A study on the relationship between chemical components of wood and their sugar released for ethanol production has been previously reported (Dwianto *et al*. 2011). The content of cellulose in wood was not consistently related to the sugar released. This trend was also witnessed in the relationship between hemicellulose and the sugar released. However, the lignin content in wood generated an expected trend whereby lower lignin content was related to higher sugar release. The complexity of wood structure and high lignin content were believed to be the major factors inhibiting contact between cellulose and the enzyme in the saccharification process. It might be true that even though the wood has a high level of cellulose, the composition of its other chemical components such as hemicellulose and lignin may act as inhibitors in the conversion of its cellulose into reducing sugar that could be further converted into ethanol.

Conclusions

In our study, *F. malayana* and *P. indicus* had yields from saccharification and ethanol production that were higher than those from sengon xylem, which is one of the mostly highly hydrolysable types of xylem in Indonesia. Despite sengon's advantageous characteristic, among the fast growing tree species analyzed from Purwodadi Botanical Garden, *F. malayana* and *P. indicus* were determined to be the best tree species for bioethanol production.

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