

## Enzymatic Saccharification of *Salix viminalis* cv. Q683 Biomass for Bioethanol Production

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**ABSTRACT** : The possibility of employing biomass of *Salix viminalis* cv. Q683 as a resource of bio-energy was evaluated. The chemical analysis of *S. viminalis* cv. Q683 leaf biomass showed components such as, extractives (2.57%), lignin (39.06%), hemicellulose (21.61%), and cellulose (37.83%), whereas, its stem was composed of extractives (1.67%), lignin (23.54%), hemicellulose (33.64%), and cellulose (42.03%). The biomass of *S. viminalis* cv. Q683 was saccharified using two enzymes celluclast and viscozyme. The saccharification of *S. viminalis* cv. Q683 biomass was influenced by enzymes and their strengths. The optimal enzyme combination was found to be celluclast (59 FPU/g substrate) and viscozyme (24 FBG/g substrate). On saccharification the glucose from leaf and stem biomass was 7.5g/L and 11.7g/L, respectively after 72 hr of enzyme treatment. The biomass and enzyme-treated biomass served as the feedstock for ethanol production by fermentation. The ethanol production from stem and leaf biomass was 5.8 g/L and 2.2 g/L respectively, while the fermentation of the enzymatic hydrolysates yielded 5 g/L to 8 g/L bioethanol in 72 hours.

**Keywords** : Bioethanol, Enzymatic hydrolysis, Fermentation, *Salix viminalis* cv. Q683, Willow

### INTRODUCTION

Bioethanol derived from plant biomass is one of many renewable energy alternatives to fossil fuels. As a substitute for gasoline, bioethanol has great potential since the distribution system for liquid fuel already exists (Sorensen *et al.*, 2007).

Lignocellulosic biomass is an inexpensive and abundant feedstock, for the economic production of bioethanol. Extensive research has been directed towards the conversion of lignocellulosic biomass to ethanol in the past two decades (Dale *et al.*, 1984; Bjerre *et al.*, 1996; Duff and Murray, 1996; Wright, 1998).

The cost of ethanol production from lignocellulosic biomass is relatively high with current technologies. The pretreatment procedures that are presently in use still leave room for improvement in efficiency and economy.

This is because these processes usually require the use of chemicals such as sulfuric acid and ammonia that enhance the costs of the process. In addition these chemicals also require a neutralization or recovery step to reduce the loads on the environment. Hence, a new pretreatment technology is required to be established for an efficient and economical ethanol production from lignocellulosic biomass.

*Salix viminalis*, the Common Osier or Osier, is a species of willow native to Europe and western Asia. *S. viminalis* is a multi-stem shrub growing to between 3–6 m. *S. viminalis* variety Q683 was developed as a bioenergy crop. The plant further forms a multi-stem plantation, with each stool producing up to 20 shoots. These clones grown as a SRC (Short rotation coppices) plantation constitute densely planted high-yielding varieties harvested on a 2-5 year cycle (usually 3 year) following coppice of

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the first establishment year's growth. *Salix* can be harvested 4–6 years after initial plantation, and thereafter on at every 3–4 years intervals for a total of 20 years or more. After the harvest, *Salix* is usually converted to wood chips to enter into the mainstream supply of forest residues (Helby *et al.*, 2006). However, this *Salix* clone has not been explored as a biomass feedstock for bioethanol production. Thus, this study was carried out to evaluate the possibility of *S. viminalis* cv. Q683 biomass as a bioethanol resource. We also report that the saccharification step using enzymes generates *S. viminalis* cv. Q683 feedstock, which when fermented by yeast provides high bioethanol yields.

## MATERIALS AND METHOD

### Raw material

*Salix viminalis* cv. Q683 was harvested at Chunnam Forest Research Institute, South Korea. The leaves and stems were chopped into small pieces and ground to 20–80 mesh size and then dried at 70°C to constant weight.

### Chemical analysis

Chemical composition of the biomass was determined according to the National Renewable Energy Laboratory analytical methods (NREL, 1996). The analysis included the determination of extractives, lignin (acid-insoluble and acid soluble), hemicellulose and cellulose.

### Pretreatment of the plant material

To evaluate the sugar productivity from *S. viminalis* cv. Q683 biomass (leaf and stem) a pretreatment procedure was adopted (Koo *et al.*, 2009). The raw material was stirred with NaOH (1%) at room temperature and autoclaved at 121°C for 15 min. Insoluble fraction was washed with distilled water to collect cellulose enzymatic hydrolysis.

### Enzymatic hydrolysis of the biomass

The enzymes used for biomass hydrolysis were cellulast, a cellulase from *Trichoderma reesei* (Novo Co., Denmark), and viscozyme, a  $\beta$ -glucosidase (Novo Co., Denmark). The enzyme treatments of pretreated cellulosic biomass (1.0 g) were undertaken in a 250 ml Erlenmeyer flask containing 50 ml of 0.1 M sodium citrate buffer (pH 4.8). Various concentrations of enzyme mixtures were added and later the reaction flask was placed on a shaking incubator (IS-97IR, Jeio-Tech Co., Korea) maintained at 50°C and 120 rpm for 72 hr. Composition of enzyme mixtures was EM-1 (viscozyme 121 FBG/g substrate), EM-2 (celluclast 15 FPU/g substrate and viscozyme 97 FBG/g substrate), EM-3 (celluclast 29 FPU/g substrate and viscozyme 73 FBG/g substrate), EM-4 (celluclast 44 FPU/g substrate and viscozyme 48 FBG/g substrate), EM-5 (celluclast 59 FPU/g substrate and viscozyme 24 FBG/g substrate) and EM-6 (celluclast 73 FPU/g substrate). Samples were withdrawn after 12, 24, 48, and 72 hr to monitor the progress of hydrolysis. The sugar was analyzed in supernatants after heat denaturing of enzymes (100°C for 10 min).

### Fermentation of saccharified hydrolysate by yeast

*Saccharomyces cerevisiae* KCCM 11215, was used throughout in the fermentation study. Inoculum required for fermentation was prepared by transferring *S. cerevisiae* to YM liquid medium (yeast extract, 3 g/L malt extract, 3 g/L peptone, 5 g/L dextrose 10 g/L) contained in 100 ml flask. The yeast was grown at 35°C on an orbital shaker for 24 h. Later an inoculum ( $1.5 \times 10^8$  cells/ml) was seeded at 5% (v/v) of fermentation broth. The fermentation flasks were incubated at 35°C for 72 h, at the same time the broth samples were collected to monitor the progress of the fermentation at regular intervals to quantify ethanol and sugars.

### Determination of sugars from saccharified biomass

After enzymatic hydrolysis the samples were centrifuged to collect supernatant. The total sugar concentration of

**Table 1.** Formula for estimation of glucose concentration on hydrolysates.

Biomass	Enzyme combination	Formula of reflectometry
Leaf	C44 FPU V48 FBG	$Y=1.8x+3.6$ ( $R^2=0.998$ )
Stem	C59 FPU V24 FBG	$Y=1.88x+3.3$ ( $R^2=0.999$ )

C:celluclast V:viscozyme, Enzyme concentration used was a one gram substrate.

Y: reflecto meter index, x: glucose concentration

the hydrolysates was determined with a reflectometer (ATAGO manual, Japan) using an internal standard, hydrolysate and citrate buffer (pH 4.8, 0.1 M) mixture. Sugar content of the hydrolyzed biomass was calculated using the formula (Table 1).

### Statistical analysis

All experiments were conducted as triplicate repetitions. Analysis of variance for variations in composition, sugar released after hydrolysis, cellulose conversion, and ethanol yields were performed using PROC GLM in SAS 9.1 software (SASs Inc., Cary, NC).

## RESULTS

### Chemical composition of raw material

Chemical analysis of stem and leaves of *S. viminalis* cv. Q683 showed significant differences in their chemical composition (Table 2). Content of total extractives from stem and leaf of *S. viminalis* cv. Q683 varied. Generally the extractives include non-structural components like waxes, fats, tannins, sugars, some resins and coloring

matters. The extractives composition of leaf was 1.5% an amount higher compared to that of stem biomass. Hence leaf biomass was rich in non-structural components like fats and resins.

Lignin component was found to be higher in leaf than stem of *S. viminalis* cv. Q683. The leaf and stem biomass contained 40.6% and 23.5% lignin, respectively. The hemicellulose content of stem and leaf was 33.6% and 21.6% respectively. Similar to hemicellulose the cellulose stem biomass was 42.0% and cellulose in leaf was 37.8%.

### Enzyme hydrolysis in pretreated biomass

Acid-alkali pretreated *S. viminalis* cv. Q683 leaf and stem biomass was subjected to enzyme hydrolysis with a mixture of cellulclast and viscozyme enzymes. The yields of glucose yield after enzymatic hydrolysis and after chemical hydrolysis of *S. viminalis* cv. Q683 biomass with NaOH are presented in Fig. 1. The effect of enzymes and their optimal concentrations of enzyme combinations for saccharification of *S. viminalis* cv. Q683 biomass were found to be cellulclast (59 FPU/g substrate) and viscozyme (24 FBG/g substrate).

**Table 2.** Chemical composition of *S. viminalis* cv. Q683 biomass.

Chemicals	Contents (g/100 g dry weight)	
	Stem	Leaf
Extractives	0.8	1.5
Lignin		
Insoluble*	59.4	75.7
Soluble	40.6	24.3
Hemicellulose	21.6	33.6
( $\alpha$ )-Cellulose	37.8	42.0

\*Insoluble as holocellulose

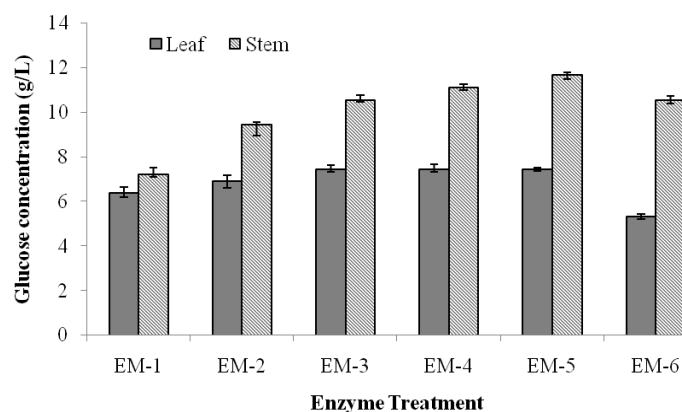
The enzyme hydrolysate showed differences in total sugars depending upon enzyme mixtures used. The enzymatic hydrolysis of stem biomass was easier than leaf biomass on a comparative basis. For the stem biomass saccharification, a mixture of celluclast (59 FPU/g substrate) and viscozyme (24 FBG/g substrate) was the best combination yielding a total sugar of 10 g/L after 72 hr of enzymatic hydrolysis. At the same time the optimal enzyme combination of enzymes required for leaf biomass saccharification was celluclast (29 FPU/g substrate) and viscozyme (73 FBG/g substrate) with a final yield of 8.5 g/L of total sugars.

The time course of enzymatic hydrolysis of stem and leaf biomass is shown (Fig. 2). The concentration of free sugars significantly increased with hydrolysis time. Initially

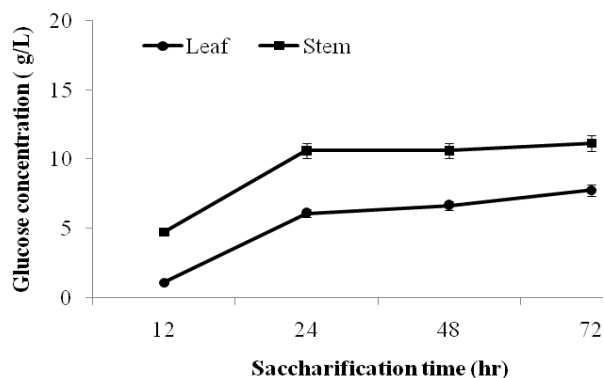
the saccharification increased until 24 hr of incubation and stabilized thereafter. Among the two biomass tested, stem showed 10 g/L digestibility while leaf was digested to the extent of 8.0 g/L.

#### Ethanol production from stem and leaf hydrolysates

The ethanol production from stem and leaf biomass was dependent on the basis of enzyme combination and their concentration (Fig. 3). The correlation of ethanol production and rate of enzyme hydrolysis was high. Production of ethanol from leaf biomass was between 2.5 g/L and 5 g/L, however ethanol production from leaf was as low as 2.5 g/L.



**Fig. 1.** Total sugar concentration after enzyme hydrolysis of *S. viminalis* cv. Q683 biomass with various enzyme mixtures. EM-1 (viscozyme 121 FBG/g substrate), EM-2 (celluclast 15 FPU/g substrate and viscozyme 97 FBG/g substrate), EM-3 (celluclast 29 FPU/g substrate and viscozyme 73 FBG/g substrate), EM-4 (celluclast 44 FPU/g substrate and viscozyme 48 FBG/g substrate), EM-5 (celluclast 59 FPU/g substrate and viscozyme 24 FBG/g substrate) and EM-6 (celluclast 73 FPU/g substrate)



**Fig. 2.** Time course of enzymatic saccharification of *S. viminalis* cv. Q683 biomass. Enzyme mixtures and pretreated biomass were incubated at 50°C and 120 rpm.

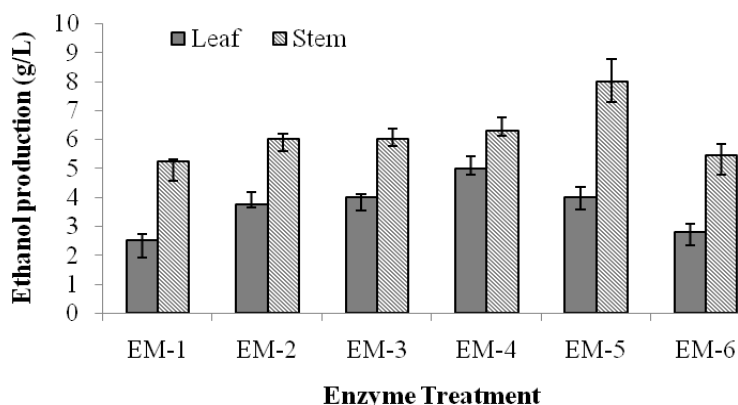
The ethanol production rate increased in the initial fermentation process which then somewhat increased until 72 hr (Fig. 4). Bioethanol production from stem enzyme hydrolysates was 5.8 g/L after 72 hr, however ethanol production from leaf hydrolysates was only 2.2 g/L.

## DISCUSSION

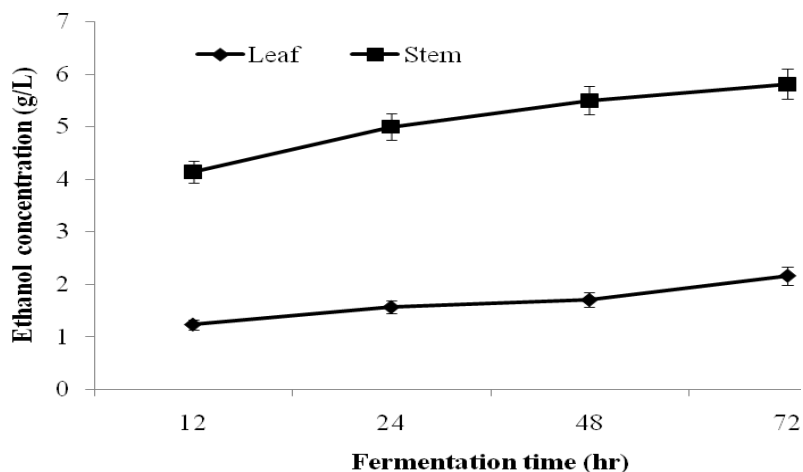
Lignin content in *S. viminalis* cv. Q683 was comparable and/or higher than that reported for other plant woods and grasses like birch, spruce, pine, switchgrass woods (Hayn *et al.*, 1993; Wiseloge *et al.*, 1996). The cellulose

content was higher than that reported for other woods like eucalyptus, aspen and spruce (Ramos *et al.*, 1992). The differences in the chemical composition and degree of hydrolysis are due to cell wall structure and compositions. The structure and composition of cell walls vary depending on plant taxa, tissue, age and cell type, and also within each cell wall layer (Bothast *et al.*, 2005; Ding *et al.*, 2006).

In this study, 1% NaOH treated in *S. viminalis* cv. Q683 biomass. This pretreatment yields low ethanol production compared to acid-alkali pretreatments (Data not shown). However, among all the pretreatment methods, alkaline pretreatment has received more attention because



**Fig. 3.** Ethanol production from enzyme hydrolysates *S. viminalis* cv. Q683 biomass. EM-1 (viscozyme 121 FBG/g substrate), EM-2 (celluclast 15 FPU/g substrate and viscozyme 97 FBG/g substrate), EM-3 (celluclast 29 FPU/g substrate and viscozyme 73 FBG/g substrate), EM-4 (celluclast 44 FPU/g substrate and viscozyme 48 FBG/g substrate), EM-5 (celluclast 59 FPU/g substrate and viscozyme 24 FBG/g substrate) and EM-6 (celluclast 73 FPU/g substrate)



**Fig. 4.** Time course of ethanol production from *S. viminalis* cv. Q683 biomass at fixed enzyme mixtures (EM-4 & EM-5) for leaf and stems.

Hydrolysates and *S. cerevisiae* broth were cultured at 35°C for 120 rpm.

it is relatively inexpensive, less energy intensive, and effective on many feedstocks especially forages and agricultural residues (Belkacemi *et al.*, 1998; Xu *et al.*, 2010). The application of alkaline solutions leads to removal of the lignin barrier, disruption of structural linkages, reduction of cellulose crystallinity, and decrease in the degree of polymerization carbohydrates (Sun and Cheng, 2008). NaOH is one of the most effective alkaline reagents and has been used to treat a variety of lignocellulosic feedstocks (Fox *et al.*, 1989; MacDonald *et al.*, 1983; Silverstein *et al.*, 2007)

Hydrolysis of sugars in *S. viminalis* cv. Q683 was affected depending on enzyme combination and its concentration. Celluclast treatment improved hydrolysis rate in leaf and stem biomass. Ethanol production also increased with high concentration of celluclast. However, viscozyme treatment in single was ineffective on hydrolysis rate and ethanol production. Combination of celluclast and viscozyme enhanced the efficiency of hydrolysis. Recent studies show the importance of new balanced enzymatic complexes containing optimal combinations to effectively modify the complex structure of lignocellulosic materials (Merino and Cherry, 2007). In this study, use of optimal enzyme combination (EM-4 and EM-5) can be accomplished complete hydrolysis of cellulose to glucose.

The plant tissue proved to be an important factor affecting ethanol production. Stem biomass was suitable in ethanol production and hydrolysis compared to leaf biomass. Annual leaf/wood production of *S. viminalis* between corresponding fertilized and control plots ranged from 0.23 to 0.39, indicating a considerable potential for increasing aboveground production in some cases (Heinsoo *et al.*, 2009).

The reports on the suitability of plants for bioenergy production from plant tissue are few. The tissues for optimal bioethanol production vary depending on plant species and harvest time. Hu *et al.* (2011) reported that the cellulose-to-glucose yield of biomass after enzymatic hydrolysis of leaves and internodes was 60.5% and 26.7% respectively. These results indicate that entire application of optimal condition as pretreatment, enzyme

hydrolysis, and ethanol fermentation process for efficient bioethanol production.

This study demonstrated that enzymatic hydrolysis of *S. viminalis* cv. Q683 was low compared to other species. The yields of ethanol were also low as the hydrolysate was dilute. However, this study indicated that stem biomass of *S. viminalis* cv. Q683 is a good biomass for beneficial saccharification with consequent bioethanol yields. Although leaves served as poor fermentation substrates for ethanol production, *S. viminalis* cv. Q683 can provide us useful biomass through SRC plantation for producing bioenergy.

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