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CONVERSION OF LEAFWASTE TO SUGAR AND ETHANOL BY SHF AND SSF FERMENTATION USING CELLULASE FROM *CELLULOMONAS* SP.

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ABSTRACT:

The study to develop high sugar production by optimization of enzymatic hydrolysis process as well as applications of enzyme for ethanol production using *Acacia auriculiformis Cunn.*'s leaves *by Saccharomyces cerevisiae* was also investigated. *Acacia auriculiformis Cunn.*'s leaves contained cellulose and lignin at 48.9±7.6% and 44.3±0.6%, respectively. However, after treated with 2% $(NH_4)_2SO_4$ for 24 h in supplemented with stream autoclave (121^oC, 15 min), giving highest content of cellulose (93.1±0.4%) while lignin content decreased significantly (6.4±1.2%). Effects of substrate concentration, cellulase concentration and incubation time on sugar production were studied. The highest sugar production at 4.5 g/L was achieved from the optimal condition containing 30 g leaves/L with cellulase activity from *Cellulomonas* TSU-03 at 1,800 U/mg protein for 16 h of incubation. Glucose was identified as a major sugar component in cellulosic hydrolyzate using HPLC analysis. The ethanol production from pretreated leaves using *S. cerevisiae* in both SHF and SSF process was 1.00 and 1.08 g/L, respectively.

Keywords: *Cellulase, Ethanol, Leaves, Sugar production*

[I] INTRODUCTION

Bioethanol is a form of alternative energy that can be produced from sugarcane, mollases and agricultural wastes [1, 2]. However, cost of raw material and cellulases in enzymatic hydrolysis are regarded as a major factor [3]. Research and development to reduce the cost of bioethanol has been carried out in various aspects. Relatively large amount of bioethanol production tried to reduced cost at substrate level by using lignocellulosic materials as substrate such as sugarcane [4], corn [5], sugar beet [6] as well as agricultural waste, wastepaper [1, 7, 8] and leaf waste [9, 10] due to there is renewable, mainly unexploited, largely abundant, inexpensive resource and high content of readily convertible and fermentable sugar [8, 11 - 13].

In this study cost of enzymatic hydrolysis and substrate were reduced by utilize of crude cellulase from *Cellulomonas* sp. TSU-03 which produced high activity of cellulase (1,860.1 U/mg protein) as reported in our previous study [14]. In addition, the leaves of *Acacia auriculiformis Cunn.*, an abundant plant found in Thailand, were selected as substrate for ethanol production. Dry leaves are municipal-residues burnt after falling and can be transformed to sugars in a short time due to the microcrystalline nature of its cellulose [15]. Utilizing these leaves would aid pollution abatement. The goal of this study aim increase in sugar yields by optimization study in lignocellulose hydrolysis by cellulase from *Cellulomonas* sp. as well as sugar hydrolyzate from leaf waste was utilized as substrate for ethanol production by both Simultaneous saccharification and fermentation (SSF) and Separate hydrolysis and fermentation (SHF) process using *Saccharomyces cerevisiae*.

[II] MATERIALS AND METHODS

2.1. Leafwaste materials

Leaf waste obtained from Thaksin University (Phattalung, Thailand) was used as substrates. The control materials were prepared as pieces of 1 cm. x 1 cm. and stored at room temperature until required [7]. Waste materials were

pretreated with various reagents including HCl, NaOH and $(NH_4)_2SO_4$ at concentration of 2% (w/v) for 24 h with/without stream autoclave (121^{o}C, 15 min). Therefore, all lignocellulosic samples were neutralized prior to utilization and partial characterization of cellulose, holocellulose and lignin content.

2.2. Compositional analysis of leafwaste materials

The content of cellulose and lignin were determined using the TAPPI Test Method [16]. Holocellulose content was determined by sodium chlorite method according to Browning [17].

2.3. Optimization for enzymatic hydrolysis using leafwaste

The experiments were carried out by using conventional technique varying one factor at a time while keeping the other factors constant.

The enzymatic hydrolysis reaction was carried out at room temperature in an orbital shaker (150 rpm) and incubated with different amounts of leafwaste (0-40 g/L) for 0-32 h of incubation. Crude cellulase derived from *Cellulomonas* sp. TSU-03 was utilized throughout this study [14].

2.4. Fermentation studies

Microorganism and cultivation medium

Flocculation yeast strain *Saccharomyces cerevisiae* TISTR 5048 (Culture collection, Thailand Institute of Scientific and Technological Research, Thailand) was used in all the cultivation experiments. The strain was maintained on yeast malt peptone (YMP) agar plates made of yeast extract 10 g/L, soy peptone 20 g/L and agar 20 g/L with D-glucose 20 g/L as an additional carbon source [18].

Starter preparation and cultivation conditions

Starter cultures were prepared by inoculation of 24 h cultures from agar slant into 100 mL nutrient broth (NB) and incubated at 35^{o}C on a shaker (200 rpm) for 48 h. The starter culture (10%) of *S. cerevisiae* (contained 1.5 x 10^4 viable cells/mL) was inoculated into NB and cultivated on a shaker (200 rpm) for 96 h.

Simultaneous saccharification and fermentation (SSF)

A steam-autoclaved (121^{o}C, 1 atm for 15 min) suspension of leafwaste in 0.05 M Tris-HCl buffer, pH 4.5 was used as SSF medium. SSF experiments were started by inoculation with yeast starter and addition of filter-sterile enzyme solution under optimum condition obtained above.

Separate hydrolysis and fermentation (SHF)

A filter-steriled hydrolyzate produced from leafwaste under optimal condition was used as fermentation medium with no supplement addition. A control fermentation was also run, on YMP medium supplemented with reagent-grade of glucose (Merck, Gemany) at the same concentrations present in the sludge hydrolyzate used for the SHF process.

2.5. Analytical method

Ethanol and sugar were measured. Triplicate samples of ethanol were analyzed by HPLC (Waters, USA) which was equipped with a reflective index (RI) detector an Aminex HPX-87H column (Bio-Rad, USA). Ethanol identification was performed with at 60^{o}C with eluent of 5 mM sulfuric acid as mobile phase at a flow rate of 0.4 mL· min [18].

Reducing sugar content in the hydrolyzate was determined quantitatively by using Nelson Somogyi method as outlined [19].

[III] RESULTS

3.1. Characteristics of *Acacia auriculiformis Cunn.*'s leaves

The basic structure of leafwaste consists of three basic polymers: cellulose, hemicellulose and lignin [14, 20]. Cellulose and hemicelluloses should be provided as precursor for fermentable sugars and ethanol product [21]. The effect of pretreated on cellulose content was also determined. Samples were pretreated with various reagents including HCl, NaOH and $(NH_4)_2SO_4$ at concentration of 2% (w/v) for 24

with/without stream autoclave (121°C, 15 min). The biochemical characteristic of leaf waste was shown in **Table-1**.

Treatments	Autoclave	Cellulose (%)	Holocellulose (%)	Lignin (%)
Before				
Leaf waste		48.9±7.6	55.7±1.0	44.3±0.6
After				
2% HCl	–	49.1±0.6	58.7±1.3	41.3±0.3
	+	85.9±0.2	86.7±3.1	13.3±0.6
2% NaOH	–	49.5±0.8	58.5±2.4	41.5±0.9
	+	88.4±1.2	88.7±1.6	11.3±2.1
2% $(NH_4)_2SO_4$	–	53.6±1.5	69.8±1.2	30.2±1.2
	+	93.1±0.4	93.6±0.7	6.4±1.2

Table: 1. Biochemical compositions of leafwaste under various pretreatment reagents with or without stream autoclave.

Different pretreatments were applied to increase the degradation activity of the cellulase from *Cellulomonas* sp. TSU-03. Steam explosion with 2% of $(NH_4)_2SO_4$ was the best pretreatment that caused significant increased cellulose (93.1±0.4%) followed by NaOH (88.4±1.2%) and HCl (85.9±0.2%). However, pretreatment with reagent without stream autoclave did not negatively affect on cellulose content.

Steam explosion is typically initiated at a temperature of 160-260°C (corresponding pressure 0.69 – 4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis [8, 22].

3.2 Optimization for enzymatic hydrolysis using leafwaste

The effect of waste concentration and incubation time was studied.

Effect of different amount of leafwaste

An initial increasing trend of sugar formation was observed when more of substrate was degrade with fixed enzyme concentration (1,800 U/mg protein). The highest reducing sugars concentration at 4.5 g/L was obtained from the optimal leafwaste at an amount of 30 g/L. In detail, the concentration of sugars was found to increase with increasing leafwaste from 2 – 30 g. Leafwaste in the range of 2 – 30 mg gave sugar amount at 0.03 – 4.5 g/L. High amounts of leaf waste tested (40 g) gave lower sugars at 1.1 g/L (**Table-2**).

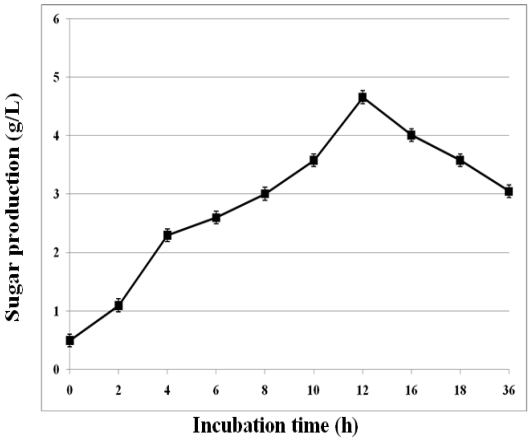
Leaf waste (g)	Sugar production (g/L)
0	0
2	0.03±0.1
3	0.11±0.1
4	0.20±0.3
5	1.10±0.1
10	2.24±0.1
20	2.99±0.2
30	4.50±0.1
40	1.10±0.3

Table: 2. Effect of different amount of leafwaste on sugar production.

Effect of incubation time

The effect of incubation time was also studied. The highest sugar production 4.66±0.3 g/L was obtained from cellulase at 1800 U/mg of protein after 16 h of incubation time **(Figure-1)**.

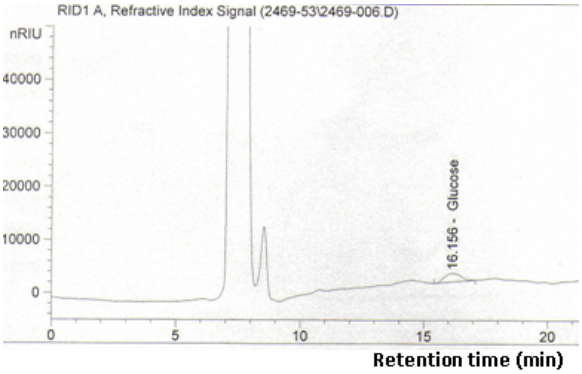
Fig: 1. Effect of incubation time (0 – 36 h of incubation) on sugar production from leafwaste (30 g/L).



3.3 Identification of wastepaper hydrolyzate composition

Cellulose, major component of leafwaste can be converted enzymatically to sugars (e.g., glucose, cellobiose, cellotriose xylose and L-arabinose, etc.) and subsequently fermented to ethanol. Therefore, sugar content obtained from leafwaste hydrolyzate was identified by HPLC analysis. Glucose was found to be a major end product (4.66 g/L) in enzymatic hydrolyzate. The present of glucose suggests the complete action of enzyme during lignocellulose hydrolysis (**Fugur2)**

Fig: 2. HPLC analysis of cell-free suspension of leaf waste hydrolysed by *cellulase* where glucose was identified at 16.156 min.



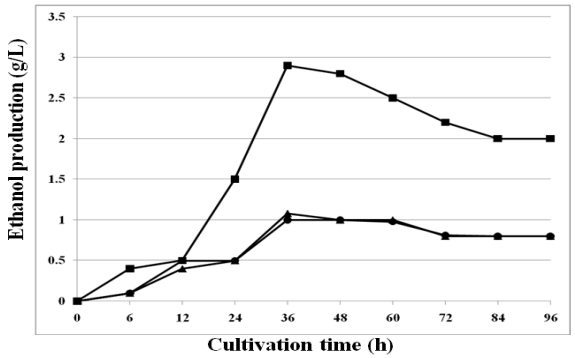
3.4 Ethanol production using leaf waste as raw substrate

Hydrolysate obtained from leafwaste under optimal condition was used as fermentation medium, with no nutritional supplementation, for the separate hydrolysis and fermentation (SHF) and sequential saccharification and fermentation (SSF) process, to produce ethanol with *S. cerevisiae* TISTR 5048.

The fermentation of the hydrolyzate (containing glucose at 4.66 g/L) was compared with that of YMP medium supplemented with reagent-grade glucose at these same concentration.

The time course profiles obtianed for both fermentation experiments are represented in **Figure-3**. The ethanol production obtained in the SSF process was 1.00 g/L followed by SHF (1.08 g/L) after 36 h of cultivation, coresponding to an ethanol volumetric production rate of 0.02 – 0.03 g ethanol/L·h. However, the highest ethanol concentration was obtained from YMP medium at 2.90 g/L.

Fig: 3. Time course of the fermentation with *S. cerevisiae*: YMP medium (■), SSF process (♦) and SHF process (●) on ethanol production.



[V] CONCLUSION

Acacia auriculiformis Cunn.'s leaves contained cellulose and lignin at 48.9±7.6% and 44.3±0.6%, respectively. However, after treated with 2% $(NH_4)_2SO_4$ for 24 h in supplemented with stream autoclave (121^oC, 15 min), giving highest content of cellulose (93.1±0.4%) while lignin content decreased significantly (6.4±1.2%). The highest sugar production at 6.7 g/L was achieved from the optimal condition containing 30 g leaves/L with cellulase activity from *Cellulomonas* sp. at 1800 U/mg protein for 16 h of incubation. Glucose was identified as a major sugar component in cellulosic hydrolyzate using HPLC analysis. The ethanol production from pretreated leaves using *S. cerevisiae* in both SHF and SSF process was 1.00 and 1.08 g/L, respectively.

FINANCIAL DISCLOSURE

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