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Ethanol Production from Sugarcane Bagasse by Means of Enzymes Produced by Solid State Fermentation Method

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***Abstract*—**Nowadays there is a growing interest in biofuel production in most countries because of the increasing concerns about hydrocarbon fuel shortage and global climate changes, also for enhancing agricultural economy and producing local needs for transportation fuel. Ethanol can be produced from biomass by the hydrolysis and sugar fermentation processes. In this study ethanol was produced without using expensive commercial enzymes from sugarcane bagasse. Alkali pretreatment was used to prepare biomass before enzymatic hydrolysis. The comparison between NaOH, KOH and $Ca(OH)_2$ shows NaOH is more effective on bagasse. The required enzymes for biomass hydrolysis were produced from sugarcane solid state fermentation via two fungi: *Trichoderma longibrachiatum* and *Aspergillus niger*. The results show that the produced enzyme solution via *A. niger* has functioned better than *T. longibrachiatum*. Ethanol was produced by simultaneous saccharification and fermentation (SSF) with crude enzyme solution from *T. longibrachiatum* and *Saccharomyces cerevisiae* yeast. To evaluate this procedure, SSF of pretreated bagasse was also done using Celluclast 1.5L by Novozymes. The yield of ethanol production by commercial enzyme and produced enzyme solution via *T. longibrachiatum* was 81% and 50% respectively.

***Keywords*—**Alkali pretreatment, bioethanol, cellulase, simultaneous saccharification and fermentation, solid state fermentation, sugarcane bagasse

I. Introduction

In the last few decades, bioethanol has assumed a very important place among renewable fuel resources and its market is continuously expanding.

Air pollution, global warming, and the future of oil production are among major causes of public and private interests in developing ethanol as an additive or substitute for oil. This is true especially when the oil peak is estimated to reach sometime between 1996 and 2035 [1].

Currently, major raw materials for production of ethanol are sugarcane juice (sucrose) in Brazil and corn (starch) in the USA. However, there is a strong argument that expansion of ethanol production requires alternative sources, such as wood and agricultural wastes. In general, Lignocellulosic biomasses are widely available in form of agricultural or forest wastes, used paper, and other municipal degradable trash [2]. One of the common characteristics of these "alternative" raw materials is they are not food, hence are not needed to be planted as sugarcane or corn also. There is a hope that large production of ethanol from Lignocellulosic biomass resource becomes a reality by 2015 [3].

Lignocellulosic biomass (LB) is mainly composed of two polymeric carbohydrates: cellulose and hemicellulose. Lignin, another constituent of LB, acts as a "skin" and prevents easy access to cellulose.

Ethanol production from LB is done through four main steps: pretreatment, hydrolysis, fermentation and recovery and purification. The aim of the pretreatment is breaking down the LB structure and preparing it for enzymatic hydrolysis. In hydrolysis, fermentable monosaccharides are produced from hydrolysis of cellulose and hemicelluloses. Hydrolysis and fermentation can perform separately or simultaneously. In Separate Hydrolysis and Fermentation (SHF) hydrolysis and fermentation is done sequentially and in Simultaneous Saccharification and Fermentation (SSF) cellulose hydrolysis and hexose fermentation is simultaneously performed.

One of the major difficulties in SSF method is the difference between the optimum temperature for Saccharification (45˚C - 50˚C) and that of fermentation (25˚C - 35˚C). SSF requires microorganisms that survive in high temperatures. Generally yeasts and bacteria are used in fermentation. *S. cerevisiae* yeast is more resistant to ethanol and other inhibitors in hydrolysate. Therefore, it is crucial to find species of this yeast that can efficiently ferment sugars to ethanol in temperatures more than 35˚C [4].

The dominant discussion over the transformation of LBs to liquid fuel is its economic feasibility. For example, the cost of cellulase enzyme and final purification is approximately 30%-50% and 20% of the total cost respectively. To produce ethanol commercially profitable form LB there is a need for technological improvement and cost reduction in all the stages of production [5].

Solid state fermentation is defined as culturing of microorganisms on a moist solid bed. This bed might be inert or insoluble substrate which is also served as carbon and energy resources [6]. The cellulase production is done by two

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methods: submerged liquid fermentation and solid state fermentation. While most researches on cellulose hydrolysis have been conducted on submerged fermentation and while this method has many advantages regarding the process control and monitoring, it is a complicated process and usually involves mixing, forced aeration and control of temperature, pH, dissolved oxygen and flow rate of gas. In contrast solid state fermentation is simpler, requires less energy, may utilize lignocellulosic materials with lower quality and is less susceptible to contamination [7]. Enzyme production by fermentation using lignocellulosic substrates is inexpensive. Therefore using strategies such as solid state fermentation is an efficient way to reduce costs of cellulase enzyme production [8]. It has been demonstrated that the performance of hydrolysis with enzymes produced on lignocellulosic materials which are to be hydrolyzed is better than that of enzymes produced on other materials, such as pure cellulose. Furthermore, enzyme production onsite results in lower expenses [9].

II. Materials and Methods

A. Raw material and pretreatment

Sugarcane bagasse (47% glucose and 23% xylose) was obtained from Furfural Company of Shoushtar. We dry the bagasse, by spreading it over a shallow bed for two days. Then it was milled to pass through mesh number 20. In order to choosing alkali agent for pretreating bagasse, 1 molar solutions of NaOH ،KOH and $Ca(OH)_2$ was tested. The experiments were done using 10% bagasse loading at 45˚C for 24 hours and 150 rpm agitation. Pretreated bagasse was then washed to make it neutralized. After that, it was dried in laboratory environment and was analyzed for its components.

For hydrolysis and SSF, we used other batch of bagasse which was pretreated by 1.5M NaOH (0.6 grams NaOH for each gram of bagasse) at 60˚C for 3 hours without continuous agitation. Similar to the last procedure, after pretreatment, bagasse was washed, dried and then it was used.

B. Microorganisms and preparation for inoculation

Fungus T. longibrachiatum PTCC 5140, was obtained from Persian Type Culture Collection and fungus A. niger and S. cerevisiae yeast were obtained from microbial bank of our laboratory. Fungi and yeast were cultured on PDA slants for 5 and 2 days respectively. The spore suspension of Fungi containing 1-1.5×107 spores/ml was used as inoculums for production of cellulase enzyme. Liquid medium for the preparation of the yeast was composed of 50 grams glucose, 5 grams yeast extract, 1 gram KH2PO4, 0.3 grams NH4Cl and 2 grams MgSO4.7H2O per liter [4]. After inoculation of the yeast from slant in this environment, it was placed in shaker at 41°C and 130 rpm for 24 hours. 2.5% (by volume) of this prepared yeast was inoculated to SSF medium for ethanol production.

C. Enzyme production

The medium composed of 2 grams KH_2PO_4, 1.4 grams $(NH_4)_2SO_4$, 0.75 grams pepton, 0.4 grams $CaCl_2.2H_2O$, 0.3 grams urea, 0.3 grams $MgSO_4.7H_2O$, 0.25 grams yeast extract and 0.005 grams $FeSO_4.7H_2O$, 0.002 grams $COCl_2$, 0.0016 grams $MnSO_4.7H_2O$ and 0.0014 grams $ZnSO_4.7H_2O$ per liter [8] was used to moisten solid bed for enzyme production. In Erlenmeyer flask, 3 grams of milled bagasse was moistened by aforementioned medium plus 1 ml of microbial suspension to achieve 80% moisture. The flask was incubated for 4 days at 30°C. Produced enzymes were extracted by citrate buffer (pH=4.8, 8.05 M). After filtering the bagasse from the mixture, the rest was centrifuged by 10000 rpm for 10 minutes at 4°C in order to separate fungus spores. The leftover liquid was the enzyme solution which was used in biomass hydrolysis and SSF experiments.

D. Biomass hydrolysis and SSF for ethanol production

Enzymatic hydrolysis of the pretreated bagasse as a substrate was done using crude enzyme solution produced by fungi. Substrate loading, duration and temperature of each experiment are reported in table III. SSF was done with 5% bagasse loading and 2.5% (v/v) yeast inoculation in 100ml closed door Erlenmeyer flasks. Total volume of the solution was 40 ml and flasks were agitated in a shaker for 72 hours at 41°C and 130 rpm.

In order to compare produced enzymes with available commercial cellulase enzymes, preceding experiments were also conducted by Celluclast 1.5L made by Novozymes. 5 grams yeast extract, 1 gram KH_2PO_4, 0.3 grams $MgSO_4.7H_2O$ per liter, and 25 FPU/(g Cellulose) of Celluclast 1.5L, and 5% pretreated bagasse was added to citrate buffer (pH=4.8, 0.05M), then 2.5% (v/v) yeast was inoculated.

Solid biomass was sterilized before hydrolysis and SSF. Enzyme solutions were not sterilizable by heating methods. Therefore, liquid phase, only in experiments conducted with Celluclast 1.5L enzyme was sterilized with solid before adding enzyme.

E. Measurement methods

Compositional analysis of raw and pretreated sugarcane bagasse was done in a similar way to that introduced by US National Renewable Energies Laboratory [10] with small changes. In the case of pretreated bagasse the procedure was performed without ethanol washing step. The overall cellulase activity was measured as filter paper activity according to Ghose method [11]. To measure carbohydrates and ethanol concentration after enzymatic hydrolysis and SSF, they were sampled and analyzed by HPLC.

III. Results and Discussion

A. Alkali pretreatment

Alkali pretreatments results show that NaOH solution has the most impact on sugarcane bagasse structure (Table I). Furthermore regarding lower molecular weight of NaOH in

comparison with KOH and $Ca(OH)_2$, using this base is equal to less consumption of material while at the same time the resulted solid has the better composition for ethanol production. Therefore, NaOH solution was chosen for pretreatment. Compositional analysis of pretreated bagasse shows that residual solid is rich in glucose. Therefore pretreatment effect in this condition is dissolution of lignin and partially hemicelluloses.

After some other experiments with NaOH, we decided to pretreating bagasse with 1.5 molar NaOH solution at 60°C for 3 hours without continuous agitation. After this pretreatment, solid composition is 58% glucose and 17% xylose.

TABLE I
EFFECT OF DIFFERENT BASIC SOLUTIONS ON SUGARCANE BAGASSE COMPOSITION

Base	Grams/grams of solid	%glucose in solid after pretreatment
NaOH	0.4	58
KOH	0.56	54
$Ca(OH)_2$	0.74	40

B. Enzyme production

Enzyme produced by T. longibrachiatum has more standard activity than A. niger and also, produced enzyme solutions had very low standard cellulase in comparison with Celluclast 1.5L (Table II). But as we will discuss later, this large difference does not mean poor performance of these enzyme solutions in hydrolyzing pretreated bagasse.

TABLE II
STANDARD ACTIVITY OF ENZYMES

Enzyme Solution	Standard activity (U/ml)[a]	Standard activity (FPU/ml)
A. niger	0.902	-
T. longibrachiatum	0.112	-
Celluclast 1.5 L	-	56.5

[a]Too less to be expressed as FPU. One unit in this case is the amount of enzyme liberating 1 μM of glucose /ml/min

C. Enzymatic hydrolysis

Hydrolyzing pretreated bagasse using *A. niger* enzyme solution was better than *T. longibrachiatum* (Table III). This trend is unlike observed standard cellulase activities. As Kable et al. argued "the choice of an enzyme preparation is more dependent on the characteristics of the substrate rather than on standard enzyme-activities measured" [12]. Hydrolysis percentages in table III show that hemicelluloses hydrolysis and xylose production by these solutions is more through compared to glucose production from saccharification of cellulose. Results show that for each type of enzyme solutions by reducing solid substrate loading and increasing hydrolysis time, glucose concentration remains constant. Glucose concentration in hydrolysate is an inhibitory factor for enzymatic hydrolysis. One can conclude from data presented in table III that in this experiments, limiting concentration of glucose in hydrolysate of *T. longibrachiatum* is about 5 mg/ml and for *A. niger* is about 10 mg/ml. Due to this fact decreasing substrate loading from 10% to 5% resulted in higher Hydrolysis percentage.

TABLE III
ENZYMATIC HYDROLYSIS OF PRETREATED BAGASSE

Enzyme solution	Solid Substrate to liquid proportion (%)	Time (hr)	Temperature (°C)	Glucose (mg/ml)	Xylose (mg/ml)	Cellulose hydrolysis (%)	Hemicellulose hydrolysis (%)
	10	48	47	5.2	6.0	9	35
T. longibrachiatum	10	48	47	10.6	9.8	18	58
A. niger	5	72	41	5.3	5.2	18	61
T. longibrachiatum	5	72	41	9.0	9.0	28	83
A. niger	5	72	41	25.5	25.5	79	114
Celluclast 1.5L							

D. Simultaneous saccharification and fermentation

In simultaneous saccharification and fermentation (SSF) by produced enzymes, ethanol concentration by *T. longibrachiatum* was more than *A. niger* (Table IV). With this point in mind that enzymatic hydrolysis by *A. niger* enzymes produced more glucose, we expected more ethanol production by this enzyme solution. In this case analysis showed glucose presence in *A. niger* fermentation medium. This amount of glucose is less than that produced in alone hydrolysis, so it is probable that one microorganism consumed it. To describe the reason, we should say that after centrifuging the mixture in enzyme extraction step, due to hydrophobic characteristics of *A. niger*, the spores float on the surface of the solution. Probably these remained spores disturbed yeast growth and metabolism. SSF by Celluclast 1.5L was also done in order to compare ethanol production yields. In order to replicate experimental conditions and make more precise comparison, this experiment was conducted by two procedures. As it was stated earlier, it is possible to sterilize solid substrate and liquid in autoclave if we use commercial enzyme. It is obvious that putting the solid-liquid mixture at high temperature and

pressure helps its digestibility after adding enzyme (procedure A). Therefore, in procedure B solid substrate and liquid were sterilized separately and were added to each other at environmental conditions just like hydrolysis experiments which done by produced enzyme solutions. We see 15% difference in ethanol production yield between these two procedures (table IV).

TABLE IV
SIMULTANEOUS SACCHARIFICATION AND FERMENTATION, SOLID SUBSTRATE TO LIQUID PROPORTION 5%, DURATION 72 HOURS, TEMPERATURE 41 °C

Enzyme solution	Glucose (mg/ml)	Ethanol (mg/ml)	Ethanol production Yield (%)
T. longibrachiatum	0	7.2	50
A. niger	5.4	0.6	4
Celluclast 1.5L (A)	0	13.6	96
Celluclast 1.5L (B)	0	11.5	81

Ethanol production yield was calculated by using theoretical ethanol that can be produced from substrate with known glucose content. Therefore this yield is the same as cellulose hydrolysis percentage, now it is possible to compare cellulose hydrolysis percent in sole hydrolysis and simultaneous saccharification and fermentation (Table V). Results show that simultaneous implementation of hydrolysis and fermentation increases hydrolysis percent of cellulose. This impact for produced enzymes is 4 times more than that for Celluclast 1.5L.

TABLE V
COMPARISON OF CELLULOSE HYDROLYSIS PERCENTAGE IN SOLE HYDROLYSIS AND SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

Enzyme Solution	Cellulose Hydrolysis (%)	SSF (%)
T. longibrachiatum	18	50
Celluclast 1.5 L	89	96

IV. CONCLUSION

Pretreatment results show that NaOH has more effect on locally obtained sugarcane bagasse structure in comparison with KOH and $Ca(OH)_2$. Considering high ethanol production yield using Celluclast 1.5L, one can conclude that alkali pretreatment with NaOH is effective and it does not produce any materials which inhibits ethanol production.

Low cellulase standard activity of produced enzyme solutions does not mean poor performance of these solutions in hydrolyzing pretreated bagasse. This result shows that there is no relationship between cellulase standard activity of enzyme solutions and their ability to hydrolyze lignocellulosic biomass. Standard cellulase activity of enzyme solution produced by *A. niger* is lower than produced enzyme solution by *T. longibrachiatum*, but cellulose and hemicellulose hydrolysis using *A. niger* enzyme solution is better than *T. longibrachiatum* enzyme solution, and also *A. niger* enzyme solution is more tolerant to produced glucose.

Ethanol production yield via *T. longibrachiatum* crude enzyme solution by SSF method is 62% of ethanol produced by commercial cellulase enzyme (procedure B). We should also emphasize that selecting SSF method with crud onsite produced enzyme solution, which has a lower enzyme activity than the commercial one, is relatively more effective in hydrolyzing cellulose to glucose.

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