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RESEARCH ARTICLE

Ethanol production from sugarcane bagasse by means of on-site produced and commercial enzymes; a comparative study

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Abstract

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 $Ca(OH)_2$ revealed that NaOH has been more effective on bagasse structure. The required enzymes for biomass hydrolysis were produced by bagasse solid state fermentation using three fungi: Trichoderma longibrachiatum, T. reesei and Aspergillus niger. Results indicated enzyme solution produced by A. niger has functioned better than the other two in cellulose conversion during sole hydrolysis. Ethanol was produced by simultaneous saccharification and fermentation (SSF) with on-site prepared crude enzyme solutions and yeast Saccharomyces cerevisiae. Here, T. longibrachiatum had the best performance in ethanol production. To evaluate this procedure, SSF of pretreated bagasse applying Celluclast 1.5L by Novozymes was also investigated. The yield of ethanol production by commercial enzyme and T. longibrachiatum enzyme solution were 81% and 52.5% respectively.

Keywords

bioethanol · sugarcane bagasse · alkali pretreatment · solid state fermentation · cellulase · simultaneous saccharification and fermentation

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1 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 amongst the major motivations leading to public and private interest in developing ethanol production as an additive or alternative to oil. This is true especially when the oil peak is estimated to reach sometime between 1996 and 2035 [1].

Currently, major raw materials for the production of ethanol are sugarcane juice (sucrose) in Brazil and corn (starch) in the US. 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 the 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. There is a hope that large production of ethanol from lignocellulosic biomass resources 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 includes four main steps: pretreatment, hydrolysis, fermentation and finally recovery and purification. The aim of pretreatment is to break down LB structure and to prepare it for enzymatic hydrolysis. In the second step, fermentable monosaccharides are produced from hydrolysis of cellulose and hemicellulose. Hydrolysis and fermentation can perform separately or simultaneously. In "Separate Hydrolysis and Fermentation" (SHF), hydrolysis and fermentation occur sequentially while in "Simultaneous Saccharification and Fermentation" (SSF), cellulose hydrolysis and hexose fermentation take place simultaneously.

SSF offers an easier operation and a lower equipment requirement than the sequential process since no hydrolysis reactors are needed. Nevertheless, one of the major difficulties with SSF method is the difference between the optimum temperature

for saccharification (45˚C-50˚C) and fermentation (25˚C-35˚C). However we can overcome this problem by exploiting microorganisms that survive at high temperatures. As we discuss later there are some strains of *Saccharomyces cerevisiae* that have this trait [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 are approximately 30%-50% and 20% of the total cost respectively. To produce ethanol economically using LBs there is a need for technological improvements and cost reduction in all the stages of the 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 also serves as carbon and energy resource [6]. Cellulase is produced via two methods: submerged liquid fermentation and solid state fermentation. While most researches on cellulose hydrolysis have been conducted on submerged fermentation and 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 through fermentation using lignocellulosic substrates is inexpensive. Therefore using strategies such as solid state fermentation is an efficient way to reduce the 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, on-site enzyme production results in lower expenses [9].

There has been a number of investigations on bioethanol production from sugarcane bagasse (2, 10, 11, 12) as well as some papers on cellulase production for application in lignocellulosic biomass hydrolysis (7, 8, 13, 14). Furthermore, Cardona and coworkers [15] have presented a thorough review on the production of bioethanol from sugarcane bagasse. The major goal of the present study is to present an efficient – yet simple and relatively inexpensive – sequence to implement each step in bioethanol production process from sugarcane bagasse that integrates the conclusions of previous studies. Our intention has not been to optimize the process. Rather, we have sought an straightforward procedure with an acceptable efficiency.

2 Materials and methods

2.1 Raw material and pretreatment

Sugarcane bagasse was obtained from "Furfural Company" of Shoushtar, Khuzestan, Iran. The bagasse had a rough composition of (on a washed and dried basis): 53% cellulose, 24% hemicellulose, 20% lignin, 2% Ash and 1% waxes based on chemical analysis. After acid hydrolysis of sugarcane bagasse and analysis using HPLC apparatus (with the following conditions in all of the cases used in this study: injection volume: 20 μL; mobile phase: HPLC graded degassed water; flow rate: 0.35 mL/min; column temperature: 70˚C; duration: 20 min), the major components of the pretreated sample were calculated as 47% glucose and 23% xylose. The bagasse was dried by spreading it to make a shallow bed for two days. Then it was milled to pass through mesh number 20.

;In order to choose alkali agent for pretreating bagasse, 1 molar solutions of NaOH, KOHand $Ca(OH)_2$ were tested. The experiments were done using 10% bagasse loading at 45˚C for 24 hours and 150 rpm agitation. Pretreated bagasse was then washed for neutralization. Afterwards, it was dried in laboratory environment and was analyzed for its components.

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

2.2 Microorganisms and preparation for inoculation

Microorganisms employed in the fermentation of sugars from lignocellulose into ethanol are principally bacteria and yeasts. The *Saccharomyces cerevisiae* yeast has proved to be more robust than bacteria, being more tolerant to ethanol and inhibitors present in hydrolysates of lignocellulosic materials [4]. Therefore, It is the most employed microorganism for fermenting the hydrolysates of lignocellulosic biomass. This yeast ferments the hexoses contained in the hydrolysate but not the pentoses [15].

Trichoderma reesei, *Trichoderma longibrachiatum* and *Aspergillus niger* are the most common fungi which are used to produce cellulase [16]. Thus in this study these fungi have been chosen for cellulase production.

Fungus *T. longibrachiatum* PTCC 5140, was obtained from Persian Type Culture Collection and fungi *A. niger* and *T. reesei* as well as yeast *S. cerevisiae* 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 1-1.5×10^7 spores/mL was used as inoculums for the production of cellulase enzyme. Liquid medium for the preparation of the yeast was composed of 50 g glucose, 5 g yeast extract, 1 g KH_2PO_4, 0.3 g NH_4Cl and 2 g $MgSO_4.7H_2O$ per liter [5]. After yeast inoculation from the slant to this environment, it was placed in shaker at 41˚C and 130 rpm for 24 hours. 2.5% (v/v) of this yeast culture was inoculated to SSF medium for ethanol production.

2.3 Enzyme production

A medium composed of 2 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 0.75 g pepton, 0.4 g $CaCl_2.2H_2O$, 0.3 g urea, 0.3 g $MgSO_4.7H_2O$, 0.25 g yeast extract and 0.005 g $FeSO_4.7H_2O$, 0.002 g $CoCl_2$, 0.0016 g $MnSO_4.7H_2O$ and 0.0014 g $ZnSO_4.7H_2O$ per liter ø9] was used to moisten the sterilized pretreated solid bed for en-

zyme production. In Erlenmeyer flask, 3 g of milled alkali pretreated bagasse was moistened by aforementioned medium plus 1 mL of microbial spore suspension (with a concentration of 10^7 spores/mL) to achieve 80% moisture (in the case of mixed cultures 0.5 mL of each microbial spore suspension was added to maintain the same spore concentration). The flask was incubated for 4 days at 30˚C. Produced enzymes were extracted by 100 mL citrate buffer (pH=4.8, 0.05 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.

2.4 Biomass hydrolysis and SSF for ethanol production

Enzymatic hydrolysis of pretreated bagasse as a substrate was performed utilizing crude enzyme solution produced by the fungi. Substrate loading, duration and temperature of each experiment are summarized in table 2. For the SSF experiments, 5 g/L yeast extract, 1 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$ as well as 5% bagasse loading plus 2.5% (v/v) yeast inoculation in 100 mL closed door Erlenmeyer flasks were applied along with the three enzyme solutions. Total volume of the solutions were 40 mL and flasks were agitated in 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 carried out by Celluclast 1.5L made by Novozymes. 5 g yeast extract, 1 g KH_2PO_4, 0.3 g $MgSO_4.7H_2O$ per liter, and 25 Filter Paper Unit (FPU)/(g Cellulose) of Celluclast 1.5L, and 5% pretreated bagasse were 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 carried out with Celluclast 1.5L enzyme was sterilized with solid before adding enzyme.

2.5 Measurement methods

Compositional analysis of raw and pretreated sugarcane bagasse were according to procedure introduced by US National Renewable Energies Laboratory [17] with small changes. In the case of pretreated bagasse the procedure was performed without ethanol washing step.

The standard cellulase activities were measured according to the standard method presented by Adney and Baker [18] and regarding formula stated by Ghose [19]. To measure carbohydrates and ethanol concentration after enzymatic hydrolysis and SSF, they were sampled and analyzed by HPLC apparatus.

3 Results and discussion

3.1 Alkali pretreatment

In the Literature, several different schemes have been tested for bagasse pretreatment. Among these, alkali pretreatments essentially aim at delignification while the most studied ones, i.e. acid pretreatments have the effect of hydrolysis as well. Since our goal is to evaluate the ability of on-site prepared enzymes to hydrolyze polysaccharides, we selected alkali pretreatment methods. Furthermore, these methods require agents that are readily available and do not need harsh condition; thus they are relatively inexpensive [15].

Alkali pretreatments results show that NaOH solution has the most impact on sugarcane bagasse structure (Figure 1). 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 showed that residual solid was rich in glucose. Therefore pretreatment effect in this condition was dissolution of lignin and partially hemicelluloses.

After some other experiments with NaOH to find optimal condition for pretreatment, we decided to pretreat bagasse with 1.5 M NaOH solution at 60˚C for 3 hours without continuous agitation. After this pretreatment, solid composition became 58% glucose and 17% xylose.

3.2 Enzyme production

Enzyme activity equals moles of substrate converted per unit time which is rate times reaction volume. Enzyme activity is a measure of the quantity of active enzyme present and is thus dependent on conditions, which should be specified. The SI unit is the katal, which is 1 mol/s, but this is an excessively large unit. A more practical and commonly used value is 1 enzyme unit (U). One U is defined as the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute (1 μmol/min). The conditions also have to be specified; one usually takes a temperature of 25˚C and the pH value and substrate concentration that yield the maximal substrate conversion rate. Enzyme activity as given in katal generally refers to that of the assumed natural target substrate of the enzyme. Enzyme activity can also be given as that of certain standardized substrates, such as filter paper as used in this study. One unit of filter paper (FPU) activity is defined as the amount of enzyme releasing 1 μmole of reducing sugar from filter paper per mL per min. Because cellulase activity in whole broths is nonlinear in regarding enzyme concentration, this atypical assay dictates dilution of the cellulase preparation to a point where 2.0 mg of reducing sugar equivalents is released in 1 h at 50˚C and pH 4.8. This amount of enzyme is defined as one filter paper unit (FPU) [20].

Enzyme produced by *T. longibrachiatum* had the highest standard activity among in-site produced enzyme solutions and also produced enzyme solutions had very low standard cellulase activity in comparison with Celluclast 1.5L (Table 1). But as we will discuss later, this large difference does not mean poor performance of these enzyme solutions in hydrolyzing pretreated bagasse.

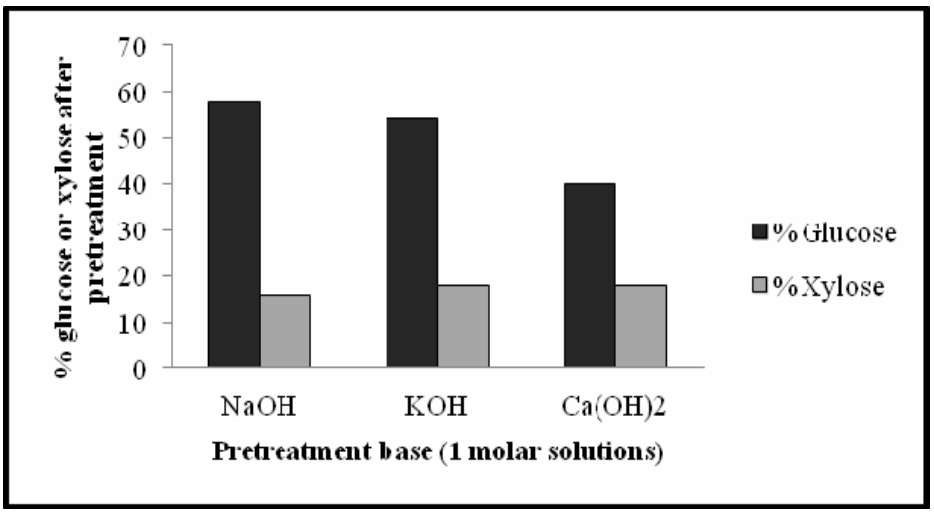

Fig. 1. Impact of pretreatment on bagasse composition

Tab. 1. Standard activity of enzymes

Enzyme Solution	Standard activity (U/mL)[1]	Standard activity (FPU/mL)
A. niger	0.0902	-
T. longibrachiatum	0.1120	-
T. reesei	0.0431	-
T. longibrachiatum + A. niger	0.0988	-
T. reesei + A. niger	0.1040	-
Celluclast 1.5 L	-	56.5

[1]1Unit/mL as defined by Ghose [19], too less to be expressed as FPU.
One unit in this case is the amount of enzyme liberating 1 μmol of glucose / min

3.3 Enzymatic hydrolysis

Hydrolyzing pretreated bagasse using enzyme solutions produced by *A. niger* (single or in combination with another fungus) were more efficient (Table 2). 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" [21]. Hydrolysis percentages in table 2 show that hemicelluloses hydrolysis and xylose production by these solutions has been more through compared to glucose production by saccharification of cellulose. It can be observed that for each type of enzyme solutions by reducing solid substrate loading and increasing hydrolysis time, glucose concentration remains constant. Hence, it is probable that glucose concentration in hydrolysate is an inhibitory factor for enzymatic hydrolysis. However, one can articulate that regarding much higher glucose concentration in commercial enzyme hydrolysate solution and unsterilized condition of prepared enzyme solutions, the reason for the low glucose concentration in the later hydrolysate solutions might be contamination. One can conclude from data presented in table 2 that in these experiments, limiting concentration of glucose in hydrolysate has been about 5 mg/mL for *T. longibrachiatum* and about 10 mg/mL for *A. niger*. Due to this fact, decreasing substrate loading from 10% to 5% resulted in higher hydrolysis percentage so as to reach the limiting glucose concentration. All in all, it is not possible to produce more glucose even by increasing time or decreasing substrate loading. Moreover, these data show that *T. reesei* has performed weakly in the saccharification of cellulose.

3.4 Simultaneous saccharification and fermentation

In simultaneous saccharification and fermentation (SSF) by produced enzymes, ethanol concentration by *T. longibrachiatum* was more than *A. niger* and *T. reesei* (Table 3). 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 which means less glucose consumption by the yeast. 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; hence some of the glucose was not used.

SSF by Celluclast 1.5L was conducted as well in order to compare ethanol production yields [22]. So as to replicate experimental conditions and make more precise comparison, preceding experiment was performed by two procedures. As 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 added to each other at environmental conditions just like hydrolysis experiments performed by produced enzyme solutions. We see 15% difference in ethanol production yield between these two procedures (Table 3).

Another thing that is worth mentioning is that *T. reesei*

Tab. 2. 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 (%)
T. longibrachiatum	10	48	47	5.2	6.0	9.4	31.0
A. niger	10	48	47	10.6	9.8	19.2	51.0
T. longibrachiatum + A. niger	10	48	47	9.7	10.9	17.5	56.5
T. reesei + A. niger	10	48	47	9.8	11.5	17.6	59.6
T. reesei	5	72	41	0.9	4.3	3.3	44.4
T. longibrachiatum	5	72	41	5.3	5.2	19.0	53.4
A. niger	5	72	41	9.0	7.1	32.6	73.0
Celluclast 1.5L	5	72	41	25.5	9.5	89.3	100.0

showed low efficiency in ethanol production via SSF just like glucose production via sole hydrolysis. As a result, it is not a good choice for either SHF or SSF.

Ethanol production yield was calculated with respect to 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 percentage in sole hydrolysis and simultaneous saccharification and fermentation (Figure 2). Results show that simultaneous implementation of hydrolysis and fermentation increases hydrolysis percent of cellulose. This impact for on-site produced enzyme is about 4.5 times more than that for Celluclast 1.5L.

4 Conclusion

It was obvious from pretreatment results that NaOH had 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. The results showed 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* was lower than that of *T. longibrachiatum*, but cellulose and hemicellulose hydrolysis using *A. niger* enzyme solution was better, and also *A. niger* enzyme solution was more tolerant to produced glucose.

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

It can be inferred from this study that selecting an efficient sequence to implement each step in bioethanol production process from bagasse may result in a high yield production procedure. It should be noted that on-site prepared enzyme solutions were not purified, i.e. enzyme was not extracted, which may affect efficiency. Further studies are required to assess other possible amendments to increase this yield. These efforts are of great importance since raw material for low-cost ethanol production is a major concern.

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Tab. 3. 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	52.5
A. niger	5.4	0.6	4.1
T. reesei	0	0.4	2.8
Celluclast 1.5L (A)	0	13.6	96.0
Celluclast 1.5L (B)	0	11.5	81.0

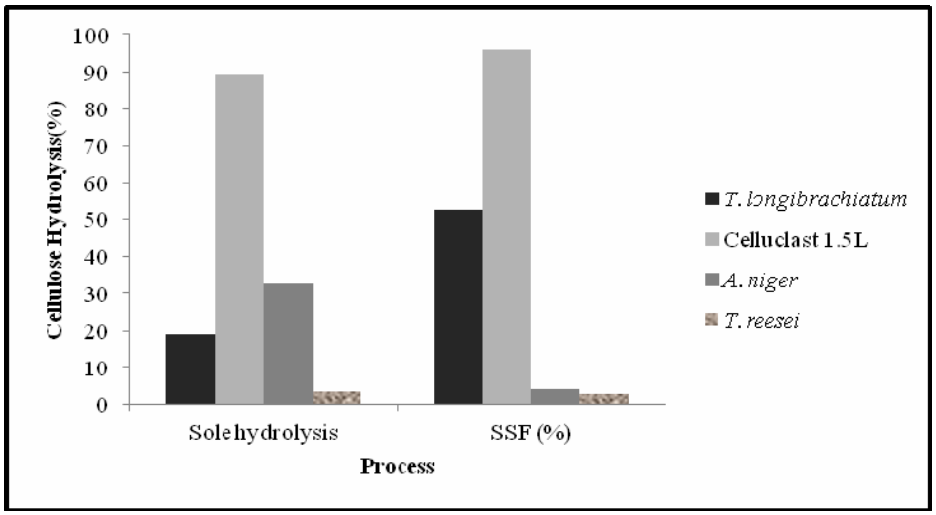

Fig. 2.

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