Simultaneous hydrolysis and fermentation of lignocellulose versus separated hydrolysis and fermentation for ethanol production

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Abstract
Second generation of biofuels relay mostly on lignocellulose as raw materials. There are several technologies applied to obtain ethanol from lignocellulose. In this study we try to compare the productivity and efficiency of two technologies: simultaneous hydrolysis ( saccharification) and fermentation (SSF) and separated hydrolysis and fermentation (SHF). We used a BlueSens equipment with CO₂ and EtOH cap sensors mounted on the fermentation flasks. In the first part we have studied how the size and type of inoculums and agitation influence the production of ethanol. In the next steps, we applied the two technologies to hydrolyze and ferment different types of cellulose from agriculture. The biomass was pretreated using alkaline and steam combined method. The cellulases used for hydrolysis are Trichoderma cellulases Onozuka from Merk, Aspergillus cellulases from Fluka, and cellobiase from Novo. The results show that the type of the yeasts is very important, especially for SSF technology, where high temperature is applied to ferment and hydrolyze in the same time. Also, the size of inoculums influence the speed of fermentation. Regarding the rate of hydrolysis and ethanol production, in case of Avicel cellulose, the hydrolysis rate is between 21 – 24% (in a fermentation medium of 10% cellulose). As for wheat straw and corn stalks, the rate of hydrolysis is much higher, over 80%. The ethanol production from lignocellulosic biomass is higher in SSF than in SHF. The results indicate that SSF is more efficient than SHF in terms of total production time, energy consumption and total production costs.

Key words: lignocellulose, hydrolysis, fermentation, ethanol

Introduction
Lately, the concentration of gases that retain parts of the radiation from the sun has increased, and the result is an increased green house effect and therefore global warming. By using bioethanol instead of petrol the emissions of green house gases decreases. The cellulose in biomass can be hydrolyzed to sugars that can be fermented into ethanol. Wheat straw and corn stalks has a rather large content of cellulose, roughly 34-45 % [1,2]. Different processes and technologies applied over the time to convert cellulose to ethanol indicate a wide range of productivity and efficiency, some authors reporting between 150 and 280 liters of ethanol / dry ton of lignocellulosic biomass [3,4,5,6]. The aims of this study were to evaluate the capacity of some local yeast strains and cellulases to work in similar conditions and to compare the productivity and efficiency of two technologies: simultaneous saccharification (hydrolysis) and fermentation (SSF) and separated hydrolysis and fermentation (SHF) applied to convert wastes from agriculture to ethanol.

Materials and methods

1. Yeasts selection. The optimal temperature for the highest enzymatic activity of cellulases was established in previous studies [7,8], and is around 50°C. Yeasts ferment sugars
at much lower temperatures. Consequently, for efficient simultaneous processes of hydrolysis and fermentation of cellulose, selection of yeasts capable to ferment sugars at higher temperatures is the first condition. In this phase, twenty-one yeast strains from the collection of industrial microorganisms of the Faculty of Animal Science and Biotechnology from Timisoara (CMIT) were tested for ethanol production at high temperatures. The yeast cultures were carried out in 100 ml flasks closed with rubber stoppers and water traps to allow CO₂ releasing. The medium used for yeast growth consists of: yeast extract 1%, peptone 2% and glucose 5%. The ethanol was determined using an enzymatic method.

2. Evaluation of ethanol production using BlueSens gas sensors. In this stage, BlueSens equipment was used to acquire data during several fermentations. The components of this system are presented in figure 1.

![Image of BlueSens equipment](image)

**Figure 1.** BlueSens equipment used to monitor fermentation

Gas counters register the volume of CO₂ released during fermentation, and sensors register the concentration of CO₂ and ethanol during fermentation. A special program – BAC Vis - transform data transmitted by sensors and counters and represent them in a plot. Fermentations were made in 1 L flasks containing 600 ml of fermentation medium (10 g yeast extract, 20 g peptone and 200 g glucose in 1000 ml water). The flasks were inoculated with different amounts of inoculums obtained with the best two yeasts (capable to produce ethanol at high temperatures) selected in the previous study: *S. cerevisiae CMIT2.8* and *S. cerevisiae CMIT2.18*. The fermentations were made at 37°C.

3. Separated hydrolysis and fermentation (SHF). The phases of this process are:

a) **Pretreatment** of lignocellulose using NaOH 2% and steam at 2 bars, for 30 minutes. After pretreatment, the liquid phase was harvested and neutralized with H₂SO₄ and the pretreated biomass was alternative washed with water and H₂SO₄ to reach pH 4.8. Around 75 and 60 grams of whet pretreated biomass was obtained from 30 grams of wheat straw and corn cobs respectively.

b) **Enzymatic hydrolysis** using Trichoderma cellulases Onozuka from Merk (15 units/gram cellulose), Aspergillus cellulases from Fluka (15 units/gram cellulose), and Aspergillus cellobiase from Novo (90 units/gram cellulose). The hydrolysis medium contains 1% yeast extract, 2% peptone, 7,5% - 10% biomass (dry weight) or cellulose Avicel, and citrate buffer pH 4.8. The nutrients will be used by yeasts in the next phase - fermentation. The pH value is...
optimal for cellulase activity. The hydrolysis is carried out at 50°C for 5 days, in a water bath with shaker. At the end of this phase samples are harvested to determine glucose concentration. 

c) Fermentation is made at 35°C with selected yeasts in a water bath with shaker, for 2-3 days. BlueSens sensors and gas counters are installed and the CO₂ and ethanol are monitored. At the end of the process the remained biomass is weighted. The whole process is carried out in 7–8 days.

4. Simultaneous saccharification (hydrolysis) and fermentation (SSF). The difference between this process and the previous is as follow: the enzymatic hydrolysis is carried out for a short period of 24 hours at 50°C, followed by inoculation and the temperature is decreased at 40°C. Afterwards the b) and c) phase from the previous processes are carried out simultaneously until the ethanol concentration is constant.

Assays.
a) The ethanol was determined in the first study, using an enzymatic method (reaction premix contains: glycine, hydrazine sulfate, Na₂EDTA, NaOH, alcohol dehydrogenase and NAD). The concentration of the resulted NADH⁺H⁺ was determined by reading OD at 340 nm.

b) A laboratory equipment, Multiparametric analyzer EOS Bravo Forte, Hospitex Diagnostics, to determine exclusively glucose. Reactive used: phosphate buffer, phenol, GOD, POD, Amino-4-antipirine. The glucose is converted in a red quinonic complex; the absorption is read at 500 nm.

Results and discussions

1. Regarding the capacity of ethanol production in yeasts, seven yeast strains with the highest capacity to produce ethanol are presented in figure 2. One yeast strain (S. cerevisiae CMIT2.18) was found to have the same ethanol producing capacity up to 40°C. Strain S. cerevisiae CMIT2.8 express the capacity to produce ethanol at high temperature as well. All yeast strains were inhibited at 45°C. Comparing the activity of cellulases and the capacity for ethanol production of yeasts, the temperature of 40°C can be used for simultaneous hydrolysis and fermentation of lignocellulose, if the yeast strain S. cerevisiae CMIT2.18 is used for fermentation of resulted glucose to ethanol.

![](image)

**Figure 2.** Capacity to produce ethanol of several yeast strains at different temperatures.
2. By using different types and quantities of inoculums to start fermentations in BlueSens equipment, results indicates a short logarithmic phase of CO₂ accumulation in the case of 2 grams / 100 ml inoculation rate – the maximal CO₂ concentration was reached in 2 hours (figure 3). In the case of using liquid culture in YPG as inoculums, the logarithmic phase of CO₂ accumulation is longer (8 hours) and the value of CO₂ concentration remains lower during the whole fermentation.

![Figure 3](image)

**Figure 3.** Plot generated by BlueSens system showing the rate of CO₂ and ethanol accumulation during fermentation.
- H13346 - sensor indicating concentration of CO₂ in the fermentation vessel with 2% inoculation rate;
- H13344 - sensor indicating concentration of CO₂ in the fermentation vessel with yeast culture in YPG;
- H13428 - sensor indicating concentration of ethanol.

Another factor studied here is agitation. The agitation timer was set for intermittent agitation (after every 15 minutes of agitation, the agitator was stopped for 15 minutes). In figure 3, the curves indicating accumulation of gas and ethanol follows the same pattern of increases and decreases of concentration. During the 15 minutes of agitation, the concentrations increases, followed by 15 minutes of decreasing during the still fermentation. Between hour 21 and 29, the agitation was made continuously and the ethanol curve indicates a continuous increasing during this period.

In this work we have studied the capacity of the two yeast strains previously selected (S. cerevisiae CMIT2.8 and CMIT2.18) to ferment glucose and produce ethanol at 40°C. In fermentation media containing 20% glucose, *S. cerevisiae* CMIT2.8 produced a maximum concentration of 16.71% ethanol, while *S. cerevisiae* CMIT2.18 produced a maximum concentration of 18.19% ethanol.

3. Regarding separated hydrolysis and fermentation (SHF) of cellulose, first we studied the capacity of the two selected yeasts to ferment glucose resulted after saccharification of pure cellulose (Avicel PH 101). The hydrolysis medium contains 10% Avicel and yeast nutrients in citrate buffer pH 4.8. The enzymatic hydrolysis was carried out for 5 days at 50°C. Figure 4 shows the pattern of CO₂ and ethanol accumulation in the vessel inoculated with *S. cerevisiae* CMIT2.18. The maximum concentration of ethanol obtained in this case is 4.132%, while in the fermentation batch inoculated with *S. cerevisiae* CMIT2.8, the maximum concentration of ethanol is 3.967%.
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Figure 4. Accumulation of CO₂ and ethanol in a fermentation process of saccharified cellulose Avicel H13346 - sensor indicating concentration of CO₂ in the fermentation vessel with *S. cerevisiae* CMIT2.18; H13344 - sensor indicating concentration of CO₂ in the fermentation vessel with *S. cerevisiae* CMIT2.8; H13428 - sensor indicating concentration of ethanol in the fermentation vessel with *S. cerevisiae* CMIT2.18.

After fermentation, the residual cellulose was harvested and the same quantities of biomass (dry weight) were obtained in both batches: 22.75 grams, resulting a hydrolysis rate of 24%.

Regarding simultaneous saccharification and fermentation (SSF) of cellulose Avicel, the maximum concentration of ethanol obtained in the fermentation batch inoculated with *S. cerevisiae* CMIT2.18 is 5.19%, while in the fermentation batch inoculated with *S. cerevisiae* CMIT2.8, the maximum concentration of ethanol is 3.85%. The residual cellulose found at the end of fermentation is 26 g for *S. cerevisiae* CMIT2.8 and 25 g for *S. cerevisiae* CMIT2.18. Although the quantity of residual cellulose is higher in this process (lower hydrolysis rate), the concentration of ethanol obtained in this case is higher.

Regarding the separated hydrolysis and fermentation (SHF) of lignocellulosic biomass (wheat straw and corn stalks), after the hydrolysis step, the concentration of glucose in the hydrolysis medium is 928.7 mg% for wheat straw and 677.3 mg% for corn stalks. The concentration of glucose in the liquid resulted after pretreatment is negligible (14.9 mg% for wheat straw and 9.2 mg% for corn stalks). For this reason, the pretreatment liquid was not added to the fermentation medium. After a fermentation of 72 hours, the concentration of ethanol (figure 5) remains low: 0.88% in wheat straw batch and 0.74% in corn stalks batch. The concentration of glucose in the fermentation media is 0.5 mg% for wheat straw and 0 mg% for corn stalks.

Figure 5. CO₂ and ethanol accumulation in separated hydrolysis and fermentation (SHF) of lignocellulosic biomass (H13344 - CO₂ sensor in wheat straw batch, H1346 - CO₂ sensor in corn stalks batch, H13428 – ethanol sensor in wheat straw)
After weighing the remained biomass after hydrolysis and fermentation, from 30 grams - the initial concentration - 8 grams and 4 grams of residual straw and corn stalks respectively has recovered. This indicates conversion rates of 73.3% in wheat straw and 86.6% in corn stalks.

![Graph showing CO2 and ethanol accumulation in simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass](image)

→Regarding simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass (wheat straw and corn stalks), after a fermentation of 48 hours, the concentration of ethanol (figure 6) is much higher than in SHF process: 1.5% in corn stalks – double (100% increasing of productivity) than in SHF in the same biomass. As for the wheat straw, the highest concentration of ethanol was 1.33%, comparing with 0.88% in SHF process, the productivity increased in this case with 50%. The concentration of residual glucose in the fermentation media is 8.2 mg% for wheat straw and 0 mg% for corn stalks. The residual biomass after fermentation was the same as in SHF process - 8 grams and 4 grams of residual straw and corn stalks respectively.

Conclusions

The results obtained in this work demonstrate that yeasts and cellulases can work in the same medium and at common parameters. Although common yeasts are able to ferment glucose and produce ethanol at 30 – 35°C, and cellulases have the optimal activity at 50°C, certain yeast strains and cellulases can act together at common temperatures of 37 – 40°C and in the same medium.

Yeast strain *S. cerevisiae* CMIT2.18 can be successfully used to ferment glucose produced by cellulases in the same medium, at temperatures of 37 – 40°C in simultaneous saccharification and fermentation (SSF) process to convert lignocellulosic biomass to ethanol.

Our results demonstrate a higher productivity of SSF process comparing with SHF process. This can be explained be inhibition of cellulase activity in the hydrolysis step of SHF process due to glucose accumulation. This inhibition can be avoided in SSF process fermenting the glucose (by yeast) simultaneously with its production (by cellulase).

High concentration of cellulose (10% in Avicel batches) lead to low conversion rates – 24%, but high ethanol concentration – over 5%. In the case of low concentration of cellulose (7.5% wheat straw or corn stalks, with a cellulose content of 30 – 35%), the conversion rates are high – over 86% in corn stalks, but concentration of ethanol is lower – 1.5% in the
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fermentation medium of corn stalks in SSF process. In large scale, high concentration of cellulose is difficult to be fermented due to mixing difficulties of dense fermentation media.

SSF is a shorter process than SHF (the highest concentration of ethanol can be reached in 48 hours in SSF, as for SHF the time for the two processes can be six days – 4 days of hydrolysis plus 2 days of fermentation). Reducing the time of a technological process more than 100% is a great achievement in industry – this will lead to low production costs. The trend in second generation of biofuels is cost reduction in order to be competitive on the fuel market, where cellulosic ethanol should replace gasoline and ethanol obtained from sugar, food or feed crops.

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References