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Microorganism and Pretreatment Effect on Lignocellulosic Bioethanol Production

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Abstract

The effects of pretreatments applied to raw materials and microorganism selection in lignocellulosic bioethanol production were investigated. It has been found that the yield of enzymatic pretreatment process applied after the chemical pretreatment is about 4 times higher than that only chemical. Enzymatic pretreatment used process yield is 3.5 times higher than that chemical pretreatment. When the microorganism ethanol production yield values of *Saccharomyces cerevisiae* and *Pichia stipitis* were examined, it was found that *S.cerevisiae* was superior to *P.stipitis* in chemical pretreated reactors (about 1.7 times higher) while *P. stipitis'* yield was higher about 1.2 times in enzymatic pretreated reactors. When the reactors which have been pretreated with both chemical and enzymatic hydrolysis and *P. stipitis* and *S. cerevisiae* used separately were examined, it was observed that there was not a great difference in terms of ethanol production yield. *C. thermocellum*'s ethanol yield was found about 3 times lower than the *S. cerevisiae* and *P. stipitis.* According to the obtained data, it was seen that *S. cerevisiae* could produce ethanol with higher efficiency than *P. stipitis*. At the same time, the difficulty of *C. thermocellum*'s production conditions, high energy demand and high risk of contamination, and low ethanol production yield, it is thought that it can only be used in the research phase for now. But in particular, by investigating extracellular cellulase enzyme system of *C. thermocellum*, genetic modifications are predicted to play an important role in the future in the second generation bioethanol production process.

1. INTRODUCTION

The largest share of primary energy supply in the world is oil and petroleum derivatives with natural gas. Petroleum-based products are a limited reserve and the only certain countries has the petroleum source. Rather, the widespread use of renewable energy resources, especially the bioethanol which can be used instead of or mix with gasoline, can contribute to economic situation and sustainability for countries that export petroleum and its derivatives. Worldwide, bioethanol production in 2016 remained at the same level as 2015 with 117.7 million m³. The fuel sector continued to account for 84% of it. World leader in the production of bioethanol is the USA with 59.5 million m³, followed by Brazil with 27.8 million m³. Total renewable energy use in the world increased by 14.42% in 2016 compared to 2015, accounting for 3% of total energy consumption. In primary energy consumption share, oil provided the largest increment to energy consumption at 77 million tonnes of oil equivalent (mtoe), followed by natural gas (57 mtoe) and renewable power (53 mtoe) [1].

It is seen that bioethanol production is mainly performed by using the products in the food chain, which is called the first generation. This situation causes problems in the social environment, especially in countries such as America where bioethanol production is intensive. In America, it is mostly organized around agri-food systems which causes crises about crops. These crises include contradictory food price and over-supply crises [2]. Converting the products that can be used as food into fuels is not welcomed, and some opposing views are being put forward. Again in America, decreased corn production and increased corn unit prices because of drought, have led to the use of corn in food production, rather than

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bioethanol production. Nonetheless, organizations that support the production of bioethanol are still being protested by American citizens [3]. For this reason, it is important to consider the use of energy plants, waste / residues of agricultural products remaining from agricultural activities and intended to be disposed of by incineration, lignocellulosic residues such as grass, grass, grass, and other aquatic plants.

The difference between first generation and lignocellulosic ethanol production is that pretreatments applied to the feedstock; lignocellulosic ethanol production's pretreatment methods are more intense and aggressive. The lignocellulosic structure needs to be decomposed to expose available/usable carbon sources, which means more energy, chemicals and time are needed than in the first generation, thus increasing the overall production costs. This is the biggest challenge for lignocellulosic ethanol production. Also, the microorganism selection is the one of the key steps of lignocellulosic ethanol production process. The selected microorganism should conform to the composition of the raw material and the optimization conditions required by the production method. The yeast and bacteria commonly used in industrial ethanol production and have advantages and disadvantages compared to each other. The most interesting of the studies carried out today is the studies on biocatalysts which can ferment the mixed sugar fractions efficiently. Some of these are *Pichia stipitis* [4], *Escherichia coli*, *Kluyveromyces* species which provides xylose fermentation and also which use the pentose like *Zymomonas mobilis*, and *Saccharomyces cerevisiae* [5-7]. In addition to these, recently, the thermophilic bacteria Clostridium thermocellum, which can metabolize the cellulose structure, and the white rot fungus, which can degrade the lignin, are also in the researches [8, 9].

Within the scope of this study, experiments have been carried out to observe how effected the lignocellulosic ethanol production by different raw materials-pretreatment methods-microorganism combinations for optimal ethanol yield.

2. EXPERIMENTAL

2.1. Microorganisms

Saccharomyces cerevisiae (DSM-1334), *Scheffersomyces stipitis* (*Pichia stipitis*) (DSM-3651), and *Clostridium thermocellum* (DSM-1237), which are obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, were used. The optical density (OD) method was used to monitor the activation of the cells.

In order to determine the density of cells; OD values were measured at 600 nm on a UV-VIS spectrophotometer (Varian Cary 50-Bio) by taking 2 ml samples at specific time intervals from free yeast and bacterial cells. The amount of dry cells was analyzed gravimetrically as a result of drying samples at different OD values at 105°C for 4 hours. Dry cell amounts corresponding to OD values were calculated using the standard curves (Figure 1, Figure 2, and Figure 3 given in Section 3).

2.2. Mediums

Yeast Extract-Peptone-Dextrose (YPD) medium was used for inoculation culture and YPD agar was used for colony forming of *S. cerevisiae* and *P. stipitis* microorganism strains. These mediums were autoclaved for 15 minutes at 121°C before cell transfer.

RM (Russian Medium) was used for the activation of *C. thermocellum* strain. While preparing RM, the following reagents and components were used in 5 septum bottles (SB).

SB-1: 10 g/l cellulose
SB-2: 2 g/l $(NH_4)_2SO_4$
SB-3: 2 g/l KH_2PO_4+ 3 g/l K_2HPO_4
SB-4: 5 g/l yeast extract
SB-5: 0.2 g/l $MgCl_2.6H_2O$ + 0.05 g/l $CaCl_2$ + 0.0025 g/l $FeSO_4.7H_2O$ + 1 g/l L- cysteine

Anaerobic environment was created by passing N_2 gas through each septum bottle to remove the oxygen present in the medium. 2 ml resazurin (1 g/l) indicator was added to the SB-3 to determine if the media was free of oxygen.

Mediums only contain physically pretreated lignocellulosic materials. In this set of experiments, it is aimed to monitor the process of thermophilic bacteria *C. thermocellum*'s extracellular enzyme system. At the same time, the amount of ethanol production from the raw materials transferred to the medium without any pretreatment was also examined.

2.3. Raw Materials

In the experiments, grass obtained from the garden of the institute, corn silage obtained from the province of Torbalı/Izmir, wheat straw obtained from the province of Tire/Izmir, MDF sawdust obtained from the institute's carpentry, and water hyacinth (*Eichhornia crassipes*) grown at the institute were used.

2.4. Pretreatment Methods

Physical, chemical, enzymatic, and microbiological pretreatments were applied to lignocellulosic raw materials.

As a physical pretreatment, the part size reduction is carried out by mechanically. It has been noted that the part size should not be greater than 1.5 cm.

As a chemical pretreatment, an alkali pretreatment which does not need any neutralization was applied. 0.5 g NaOH per g of sample was added and incubated for 24 hours at 150 rpm and 25°C [10]. HPLC (Agilent 1260 Infinity) was applied to the samples to measure carbon source quality and quantity. This application has been given the name "C" (chemical) subgroup.

As an enzymatic pretreatment, a cellulase enzyme (Sigma-Aldrich) which has been obtained from *Aspergillus niger* and the activity was 1.13 U/mg. 1.77 g of the cellulase enzyme was dissolved in pure water to get 500 ml of the enzyme solution, followed by adjusting the pH to 4.8 with citrate buffer. 100 ml enzyme solution per gram was added to the samples and incubated at 50°C in a shaking incubator at 150 rpm for 72 hours. This application was given the name "E" (enzymatic) subgroup.

As a microbiological pretreatment, *Clostridium thermocellum* microorganism was transferred to medium containing only reduced particle size lignocellulosic raw material. This application was given the name "M" (microbiological) subgroup.

Furthermore, a subgroup of "CE" (chemical-enzymatic) has been given to the application in which both chemical and enzymatic pretreatment is performed.

2.5. Analytic Methods

After the hydrolysis of the raw materials used, during the ethanol production process and at the end of the fermentation, the samples have been taken from the mediums and analyzed by HPLC to determine carbohydrate composition, by-products and ethanol concentration. The HPLC analyzes were carried out by using Aminex HPX-87H column. HPLC method and calibration has been carried out by the instructions from National Renewable Energy Laboratory (NREL) procedure. In this analysis, 0.005 M sulfuric acid was used as mobile phase and temperatures of column and RI dedector was 65°C and 35°C, respectively. The flow rate was 0.6 ml/minute and sample volume was 10 µl. 5 pointed calibration table was prepared for D-cellobiose, D(+)glucose, D(+)xylose, L(+)arabinose, glycerol, acetic acid, and ethanol (Table 1). We did not calibrate the method for 5-hydroxy-2-furaldehyde (HMF), because we did not use any of acid pretreatment. HPLC analysis' retention time was 24 minutes.

Table 1. *Concentration ranges for HPLC calibration standards*

Components	**Concentration Range (mg/ml)**
D-cellobiose	0.5–30.0
D-(+) glucose	0.5–30.0
D-(+) xylose	0.5–30.0
L-(+) arabinose	0.5–30.0
Glycerol	0.2–8.0
Acetic acid	0.2–12.0
Ethanol	1–15.0

The products to be analyzed on HPLC were first centrifuged, then passed through a 0.20 μm injector filter. Each sample was passed through separate filters. HPLC analysis was performed on each sample, taking approximately 2 ml of sample after filtration.

In order to evaluate the results obtained at the end of the fermentation, the carbohydrate (ch) consumption efficiencies of each sugar (glucose, cellobiose, xylose, arabinose) were calculated by comparing the initial sugar concentration in the medium as the substrate with the final sugar concentration. Used equations are given below [11].

$$\text{ch Cons. Yield } (\%) = (\Delta C_{ch}/C_{ch0}) \times 100 \quad (1)$$

$$\text{Max. Ethanol } (\text{v/v}\%) = C_{emax}/0.789 \quad (2)$$

$$\text{Eth. Prod. Rate } (\text{g/l. h}) = C_{emax}/t_{max} \quad (3)$$

$$\text{Microorg. Eth. Yield } (\%) = \frac{C_{emax}}{C_{ch0} - C_{chmax} \times 0.511} \times 100 \quad (4)$$

It is known that all the sugars obtained after hydrolysis do not turn into ethanol, and the cells still carry out carbohydrate destruction to form new biomass. Therefore, the comparison of initial concentrations and final concentrations of sugars to the produced ethanol concentration gives ethanol production efficiency and is calculated using the following formula based on the amount of sugar consumed [11].

$$\text{Eth. Yield}^{1}\ (\%) = \frac{C_{emax}}{(C_{glu0} + C_{cello0} + C_{xyl0} + C_{ara0}) - (C_{gluf} + C_{cellof} + C_{xylf} + C_{araf})} \quad (5)$$

$d_{ethanol}$ = 0.789 g/ml
C_{ch0} = Initial total carbohydrate concentration (g/l)
ΔC_{ch} = Initial and final carbohydrate concentration difference (g/l)
$C_{e\ max}$ = maximum ethanol concentration (g/l)
t_{max} = the time the max. amount of ethanol was detected (hour)
$C_{ch\ max}$ = Carbohydrate concentration at tmax (g/l)
C_{glu0}; C_{cello0}; C_{xyl0}; C_{ara0}= Initial glucose, cellobiose, xylose, and arabinose concentration, respectively (g/l)
C_{gluf} ; C_{cellof} ; C_{xylf} ; C_{araf}= Final glucose, cellobiose, xylose, and arabinose concentration, respectively (g/l)
0.511 = Theoretically (100%) the conversion rate of hexoses and pentoses to ethanol

[1] Arabinose concentration did not count in for the Set II's ethanol yield calculation (*P. stipitis* does not metabolize arabinose to produce ethanol, only uses for cell regeneration [12])

2.6. Experimental Sets

Since the experiments were carried out with three different microorganisms, they have been named by Set I (*Saccharomyces cerevisiae*), Set II (*Pichia stipitis*) and Set III (*Clostridium thermocellum*) and also according to the pretreatment methods they have been subdivided as C, E, CE and M (Table 2).

Table 2. *Bioprocess types, raw materials and pretreatment methods by Sets*

		Set I	**Set II**	**Set III**
	Raw material			
Group C	Grass	X	X	
	Corn silage	X	X	
	MDF sawdust	X	X	
	Wheat straw	X	X	
	Water hyacinth	X	X	
Group E	Grass	X	X	
	Corn silage	X	X	
	MDF sawdust	X	X	
	Wheat straw	X	X	
	Water hyacinth	X	X	
Group CE	Grass	X	X	
	Corn silage	X	X	
	MDF sawdust	X	X	
	Wheat straw	X	X	
	Water hyacinth	X	X	
Group M	Grass			X
	Corn silage			X
	MDF sawdust			X
	Wheat straw			X
	Water hyacinth			X

In experiments, 5 different biomass resources; grass, corn silage, wheat straw, MDF sawdust, and water hyacinth (*Eichhornia crassipes*), were used for Set I, II and III with 4 different pretreatment methods (only chemical, only enzymatic, chemical and enzymatic, and microbiological). Also, 3 different type of microorganism were used; *S. cerevisiae*, *P. stipitis*, and C. *thermocellum*. In all Sets, experiments have been carried out by three parallels. Data obtained from all batch reactors were investigate and average of those three reactors have been taken into considered. A total of 105 batch reactors were examined.

In Set I, in which *S. cerevisiae* was used, C, E, and CE pretreatments were applied. Cells were transferred to YPD medium prepared for the activation of microorganisms in Set I, then incubated for 24 hours at 25°C and 150 rpm orbital shaker incubator. 72 hours incubation for ethanol fermentation in Set I was performed.

In Set I, ethanol production was performed by separate hydrolysis and fermentation (SHF) method.

In Set II, where P. stipitis was used, C, E, and CE pretreatments were applied. In Set II, cells were incubated for 48 hours at 30 ° C and 175 rpm in an orbital shaker incubator in YPD medium for cell activation. In this set it took 72 hours for ethanol fermentation.

In Set II, ethanol production was carried out by separate hydrolysis and fermentation (SHF) as in Set I.

Grass (garden residues), wheat straw, corn silage, water hyacinth and sawdust were used in Set III where *Clostridium thermocellum* was used. Wheat straw, water hyacinth and garden wastes are mechanically reduced to the longest piece of 1.5 cm. MDF sawdust and corn silage were used directly. No enzymatic or chemical pretreatment was applied in this experimental set. The reactors transferred directly to the cell medium without pretreatment. Also in this set of experiments, the blind reactor (M_0) only containing cellulose (Fluka), was used in order to quantitatively observe the activity of *C. thermocellum*.

In Set III, RM medium was prepared for microorganism activation and incubated under anaerobic conditions at 55°C for 72 hours in a 130 rpm orbital shaker incubator. In Set III, incubation was carried out for 10 days at 55°C and 100 rpm for ethanol fermentation.

In Set III, ethanol production was carried out by the consolidated bioprocessing (CBP) method.

Set III calculations were carried out by analyzing the amount of ethanol obtained from the M_0 reactor which only cellulose used, and the amounts of ethanol obtained from the other reactors at the end of the fermentation period of 10 days.

3. RESULTS AND DISCUSSION

The figures show optical density of the microorganisms used in experiments.

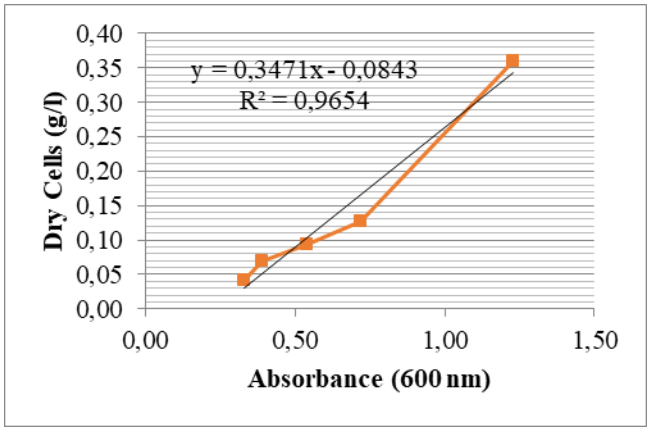

Figure 1. *OD values of S. cerevisiae*

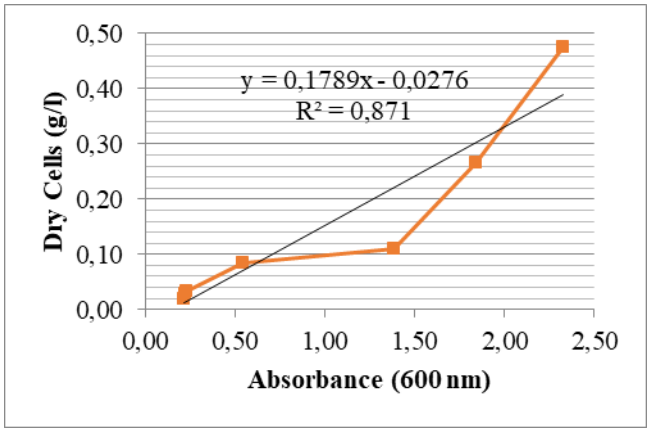

Figure 2. *OD values of P. stipitis*

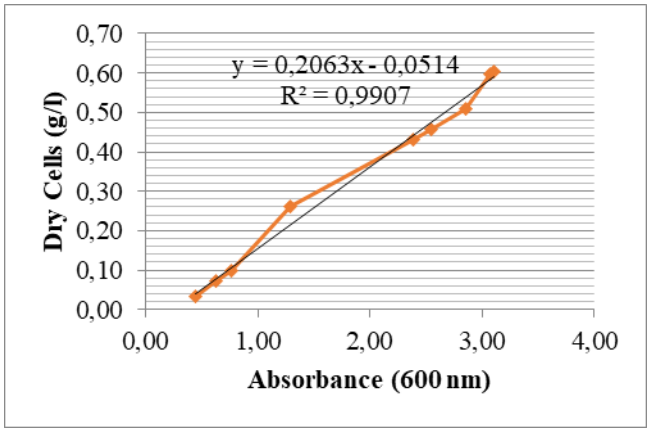

Figure 3. *OD values of C. thermocellum*

Chemical (C), enzymatic (E), and chemical+ enzymatic (CE) pretreatment's carbohydrate qualitative and quantitative results are given in Table 3, 4, and 5, respectively.

Table 3. *HPLC analysis results after chemical pretreatment*

Group C	**Cellobiose (mg/ml)**	**Glucose (mg/ml)**	**Xylose (mg/ml)**	**Arabinose (mg/ml)**	**Total Carb. (mg/ml)**
Grass	-	1.0032	1.07356	-	2.07676
Corn silage	-	1.05387	1.12087	-	2.17474
MDF sawdust	-	1.01765	1.0715	-	2.08915
Wheat straw	-	1.02958	1.0856	-	2.11518
Water hyacinth	-	1.16120	1.11275	-	2.27395

As seen from the Table 3, C group reactor does not include cellobiose and arabinose. According to a study carried out by Zhao et al. [13], it is not possible to obtain cellobiose by only using NaOH. In the study, they found out the necessity of ammonium or enzyme with NaOH to obtain soluble or usable cellobiose hydrolysis.

According to Li et al. [14], the arabinose in the xylan cannot be obtained with the pretreatment using only NaOH. For this, the hemicellulosic structure has to be degraded and enzyme hydrolysis has to be applied.

In addition, according to the results of the study, it is stated that arabinose could be partially amorphous form of cellulose, therefore, enzymatic pretreatment must be used for hydrolysis.

Table 4. *HPLC analysis results after enzymatic pretreatment*

Group E	**Cellobiose (mg/ml)**	**Glucose (mg/ml)**	**Xylose (mg/ml)**	**Arabinose (mg/ml)**	**Total Carb. (mg/ml)**
Grass	1.26322	3.95031	1.89598	1.16445	8.27396
Corn silage	1.23541	3.42195	1.10201	1.34512	7.10449
MDF sawdust	1.21532	2.86122	0.95411	1.24315	6.27380
Wheat straw	1.24278	6.17442	1.11054	1.0218	9.54954
Water hyacinth	1.11027	3.96254	1.11275	1.10156	7.28712

In group E, all kind of carbohydrates were obtained (Table 4). According to Verardi et al. [15], the use of cellulase enzymes is 30% higher than that of acid or alkaline pretreatments, especially in the presence of glucose, xylose and arabinose in the hemicellulosic structure.

The enzymatic pretreatment with the cellulase enzyme showed that cellebiose and arabinose sugars were obtained in group E compared with group C. In addition, the concentrations of glucose and xylose are higher in group E than group C.

Table 5. *HPLC analysis results after chemical+enzymatic pretreatments*

Group CE	**Cellobiose (mg/ml)**	**Glucose (mg/ml)**	**Xylose (mg/ml)**	**Arabinose (mg/ml)**	**Total Carb. (mg/ml)**
Grass	1.31679	4.27105	2.51223	1.22279	9.32286
Corn silage	1.26887	4.50531	2.16919	1.30482	9.24819
MDF sawdust	1.34731	3.27105	2.12904	1.22279	7.97019
Wheat straw	1.25457	10.09074	1.58993	1.14218	14.07742
Water hyacinth	1.13270	5.94051	1.30854	1.04376	9.42551

As a result of the alkaline and enzymatic pretreatments applied to the CE group reactors, it was determined that the concentrations of glucose and xylose were the only significantly higher than the C and E group reactors (Table 5). In the other hand, it has been seen that arabinose and cellobiose concentration is not quite different from group E reactors. Xu et al. [16] reported that sugar groups obtained with lignocellulosic feedstock degradation of cellulosic crystal structure after pretreatment with NaOH and cellulase enzyme are xylose and glucose where other sugars such as arabinose, mannose, and cellebiose obtained in lower concentrations.

It is also thought that the reason why the xylose concentration is higher than the other two groups is due to the high degree of degradation of hemicellulose to xylooligosaccharides due to the combined use of NaOH and enzymatic hydrolysis [17].

Calculations belong to Set I, Set II, and Set III are given in Table 6, 7, and 8, respectively.

Table 6. *Carbohydrate consumption (ch cons.) and yield calculations for Set I after fermentation*

	ch Cons.Yield (%)	**Max. Ethanol (v/v%)**	**Ethanol Prod. Rate (g/l.h)**	**Microorg. Ethanol Prod. Yield (%)**	**Ethanol Yield (%)**
C_{grass}	47.31	0.45	0.0049	70.83	36,19
C_{silage}	32.14	0.30	0.0033	67.34	34,41
$C_{sawdust}$	46.34	0.43	0.0047	69.02	35,27
C_{straw}	45.48	0.44	0.0049	71.09	36,33
$C_{hyacinth}$	50.63	0.55	0.0061	74.07	37,85
E_{grass}	48.09	2.37	0.0260	91.92	46,97
E_{silage}	54.28	1.58	0.0173	63.23	32,31
$E_{sawdust}$	47.59	1.56	0.0171	80.53	41,15
E_{straw}	69.58	3.86	0.0423	89.69	45,83
$E_{hyacinth}$	53.45	2.46	0.0269	97.40	49,77
CE_{grass}	47.09	2.52	0.0276	88.68	45,31
CE_{silage}	48.89	2.60	0.0285	88.80	45,38
$CE_{sawdust}$	39.90	1.96	0.0214	94.96	48,52
CE_{straw}	59.17	4.25	0.0466	78.85	40,29
$CE_{hyacinth}$	60.74	3.19	0.0350	86.11	44,00

Table 7. *Carbohydrate consumption and yield calculations for Set II after fermentation*

	ch Cons.Yield (%)	**Max. Ethanol (v/v%)**	**Ethanol Prod. Rate (g/l.h)**	**Microorg. Ethanol Prod. Yield (%)**	**Ethanol Yield (%)**
C_{grass}	91.26	0.47	0.0052	38.40	19.62
C_{silage}	59.99	0.39	0.0043	46.73	23.88
$C_{sawdust}$	84.87	0.41	0.0045	35.66	18.22
C_{straw}	80.39	0.43	0.0048	39.38	20.12
$C_{hyacinth}$	82.21	0.52	0.0057	42.76	21.85
E_{grass}	55.14	2.27	0.0249	89.46	45.71
E_{silage}	39.87	1.47	0.0161	98.63	50.40
$E_{sawdust}$	49.34	1.57	0.0173	97.96	50.06
E_{straw}	58.22	2.93	0.0321	91.12	46.56
$E_{hyacinth}$	62.73	2.40	0.0263	95.61	48.86
CE_{grass}	57.99	2.49	0.0273	81.83	41.82
CE_{silage}	64.31	2.39	0.0262	72.14	36.86
$CE_{sawdust}$	49.90	1.93	0.0212	88.52	45.23
CE_{straw}	46.87	3.33	0.0365	84.78	43.32

$CE_{hyacinth}$	39.98	2.13	0.0233	98.06	50.11

Table 8. *Ethanol production rate for Set III after fermentation*

	Max. Ethanol (v/v%)	**Ethanol Prod. Rate (g/l.h)**	**Comparison with M_0 (%)**
M_0	0.77	0.0025	
M_{grass}	0.19	0.0006	24.58
M_{silage}	0.32	0.0010	41.43
$M_{sawdust}$	0.23	0.0007	29.52
M_{straw}	0.31	0.0010	40.10
$M_{hyacinth}$	0.28	0.0009	36.80

When the qualitative and quantitative values of carbohydrates obtained after physical, chemical, enzymatic pretreatments applied to raw materials were taken into consideration, the lowest carbohydrate concentration was observed in the group C experimental set. In this group only low concentrations of glucose and xylose were found. The highest glucose concentration for group C was obtained from water hyacinth with 1.16 g/l and the highest concentration of xylose was obtained from corn silage with 1.12 g/l.

The highest carbohydrate concentration was obtained from the group CE. In this set where both the chemical and enzymatic pretreatment steps are carried out, glucose, xylose, arabinose, and cellebiose were obtained.

In general, glucose and xylose concentration values in CE reactors were obtained higher than the C and E reactors. According to Delgenes et al. study [18], it is thought that the degradation of cellulose crystal structure by pretreatment of applied NaOH and cellulase enzymes. It is also predicted - according to similar results with Li et al. [14] - that NaOH and the subsequent enzymatic hydrolysis may have resulted in higher xylose concentrations than the other two groups due to the high degree of degradation of the hemicellulosic xylooligosaccharides. The highest glucose concentration was obtained from CE group's wheat straw at 10.10 g/l.

It has been found that the yield of the enzymatic pretreatment process applied after the chemical pretreatment is about 4 times higher than that only chemical pretreatment treated process. Also, it has been seen that only enzymatic pretreatment used process yield is 3.5 times higher than that only chemical pretreatment.

In Set I and Set II, the highest consumption of carbohydrate source was monitored from CE_{wheat} with 8.33 g/l while the most bioethanol was obtained from the same reactor (3.36 g/l).

Comparison of theoretic and produced ethanol according to carbohydrate consumption for Set I and Set II is given Table 9.

Table 9. *Compare of theoretic and produced ethanol according to carbohydrate consumption for Set I and Set II*

	SET I								
	C_{ch0} (g/l)	**C_{chf} (g/l)**	**ΔC_{ch} (g/l)**	**Theoretical ethanol concentration (g/l)**	**Produced ethanol (g/l)**	**Theoretical / produced ethanol yield (%)**	**C_{ch0} (g/l)**	**C_{chf} (g/l)**	**ΔC_{ch} (g/l)**
C_{grass}	2.07676	1.09430	0.98246	0.50204	0.35557	70.83	2.07676	0.18159	1.89517
C_{silage}	2.17474	1.47588	0.69886	0.35712	0.24049	67.34	2.17474	0.87010	1.30464
$C_{sawdust}$	2.08915	1.12106	0.96809	0.49469	0.34146	69.02	2.08915	0.31602	1.77313
C_{straw}	2.11518	1.15326	0.96192	0.49154	0.34944	71.09	2.11518	0.41489	1.70029
$C_{hyacinth}$	2.27395	1.12266	1.15129	0.58831	0.43526	73.98	2.27395	0.40446	1.86949
E_{grass}	8.27396	4.29475	3.97921	2.03338	1.86912	91.92	8.27396	4.34032	3.93364
E_{silage}	7.10449	3.24843	3.85606	1.97045	1.24587	63.23	7.10449	4.78692	2.31757
$E_{sawdust}$	6.27380	3.28819	2.98561	1.52565	1.22854	80.53	6.27380	3.76579	2.50801
E_{straw}	9.54954	2.90491	6.64463	3.39541	3.04529	89.69	9.54954	4.55714	4.99240
$E_{hyacinth}$	7.28712	3.39194	3.89518	1.99044	1.93862	97.40	7.28712	3.24005	4.04707
CE_{grass}	9.32286	4.93300	4.38986	2.24322	1.98924	88.68	9.32286	4.31436	5.00850
CE_{silage}	9.24819	4.72662	4.52157	2.31052	2.05185	88.80	9.24819	4.11561	5.13258
$CE_{sawdust}$	7.97019	4.79039	3.17980	1.62488	1.54295	94.96	7.97019	4.36939	3.60080
CE_{straw}	14.07742	5.74741	8.33001	4.25664	3.35625	78.85	14.07742	7.93513	6.14229
$CE_{hyacinth}$	9.42551	3.70066	5.72485	2.92540	2.51894	86.11	9.42551	6.06276	3.36275

As can be seen in Table 9, in Set I, *Saccharomyces cerevisiae* used, when the conversion efficiency of glucose and xylose to ethanol obtained only from the chemical pretreatment (group C) is compared with the theoretical ethanol production potential, and 1.7 times higher than the Set II, *Pichia stipitis* used. This situation changes in group E reactors. It is seen that the efficiencies of the reactors of the Set II group are about 1.1 times higher when the theoretical ethanol and actual ethanol concentrations of the reactors in this group, where only the enzymatic pretreatment is used, are compared. However, in group CE, Set I values are still higher than Set II. In this case, it appears that the sugar metabolism of *S. cerevisiae* is higher than that of *P. stipitis*, and the hydrolyzed sources of carbohydrates can be transformed in a more efficient rate [19,20]. It is also seen that glucose concentrations obtained from group E reactors are lower than those obtained from group CE. The theoretical/produced ethanol yield calculated to be higher in Set II E reactors is thought to be due to the fact that *P. stipitis* can metabolize xylose higher than *S. cerevisiae* [19].

The highest amount of carbohydrate source consumed in the Set I and Set II reactors was observed to be 8.33 g/l and 6.14 g/l, respectively, in the CE_{wheat} reactors. The highest bioethanol concentration was observed at 3.36 g/l and 2.63 g/l, respectively, also in the CE_{wheat} reactors.

When the ratio of the produced bioethanol concentration and the consumed carbohydrate concentration is taken into account, the highest yields were obtained from $E_{hyacinth}$ reactors with 49.77% in Set I and 50.40% in Set II E_{silage}.

When the microorganism ethanol production yield values of Set I and Set II were examined, it was found that *S. cerevisiae* was superior to *P. stipitis* in C reactors (about 1.7 times higher) while *P. stipitis*' yield was higher about 1.2 times in E reactors. However, according to the data obtained from the CE reactors, the ethanol production efficiency of both microorganisms was found to be almost the same.

When the results obtained from the Set III reactors are examined, it can be seen that the highest bioethanol concentration is obtained from the M_{silage} reactor at a value of 0.32 g/l. It was also determined that the highest yield was again attributed to the M_{silage} reactor (M_{silage}/M_0 = 41,43%) when the amount of ethanol obtained from the cellulose-only reactor and the ethanol obtained from other reactors were compared.

When the ethanol production efficiencies were examined in general, no significant difference was observed between Set I and Set II except C reactors. It has been determined from these two microorganisms that there is no significant difference in ethanol production yield that the use of *S. cerevisiae* instead of *P. stipitis* is more advantageous for the production of second generation (lignocellulosic) bioethanol in all doses of light, In this case it has been found that *S. cerevisiae*, which is used in traditional productions such as bread-brewing and beer production, which has been called "standard" in mesophilic and contemporary works, can be used very easily and with high efficiency in the second generation ethanol production if pretreatment process carried out at appropriate conditions to obtain usable carbohydrates. It is thought that the use of *P. stipitis* in combination with *S. cerevisiae* in the continuous processes of lignocellulosic ethanol production is advantageous in that xylose obtained from pretreatments is converted to ethanol with glucose in high yield.

One of the major disadvantages of second generation bioethanol production is the high cost of pre-treatment. The extra energy, raw material and time used in the pretreatment steps have the highest share in the total cost of lignocellulosic ethanol production. For this reason, it is necessary to minimize the cost of pretreatment in order to ensure competitiveness in the market. However, for this approach to be economically feasible, an industrially relevant CBP microorganism is required that produces a hydrolytic enzyme system capable of solubilizing a realistic biomass substrate and fermenting both hexose and pentose sugars to ethanol such as *C. thermocellum* [21].

Clostridium thermocellum used in Set III have the following problems: difficulty of inoculation and ethanol production conditions, high amount of energy requirement, high risk of inhibition due to contamination, alcohol fermentation metabolism is about 3 times longer than *S. cerevisiae* and *P. stipitis*,

and low ethanol production yield. These results shows that it can only be used in research phase in the production of second generation bioethanol. However, this bacterial strain is thought to play an important role in the future, especially in the second generation bioethanol production process with genetic modifications, especially by investigating the extracellular cellulase enzyme system [22].

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

REFERENCES

[1] Renewable Fuels Assc., "2016 ethanol industry outlook, fueling high octane future", Renewable Fuels Association, web link: http://www.ethanolrfa.org/wp-content/uploads/2016/02/Ethanol-Industry-Outlook-2016.pdf, (2016). (Last access: Dec. 2017).

[2] Gillon, S., "Flexible for whom? Flex crops, crises, fixes and the politics of exchanging use values in US corn production", The Journal of Peasant Studies, 43(1):117-139p, DOI: 10.1080/03066150.2014.996555, (2016).

[3] Elmarzougui, E., Larue, B., "On the evolving relationship between corn and oil prices", Agribusiness, 29(3):344-360, (2013).

[4] du Preez, J.C., Bosch, M., Prior, B.A., "The fermentation of hexose sugars and pentose sugars by *Candida shehatae* and *Pichia stipitis*", Applied Microbiology and Biotechnology, 23:228-233, (1986).

[5] Jeffries, T., Jin, Y.S., "Metabolic engineering for improved fermentation of pentoses by yeasts", Applied Microbiology and Biotechnology, 63:495–509, (2004).

[6] Goncalves, F.A., Ruiz, H.A., dos Santos, E.S., Teixeira, J.A., de Macedo, G.R., "Bioethanol production by *Saccharomyces cerevisiae*, *Pichia stipitis* and *Zymomonas mobilis* from delignified coconut fibre mature and lignin extraction according to biorefinery concept", Renewable Energy, 94:353-365, (2016).

[7] Kasavi, C., "A system based rational approach to improve first and second generation bioethanol production by *Saccharomyces cerevisiae*," PhD Thesis, Boğaziçi University Chemical Engineering, İstanbul, Turkey, (2013).

[8] Arora, R., Behera, S., and Kumar, S., "Bioprospecting thermophilic/thermotolerant microbes for production of lignocellulosic ethanol: A future perspective," Renewable and Sustainable Energy Reviews, 51:699-717, (2015).

[9] Saddler J. N. and Chan, M. K. H., "Optimization of *Clostridium thermocellum* growth on cellulose and pretreated wood substrates," European Journal of Applied Microbiology, 16(2-3):99-104, (1982).

[10] Sills, D. and Gossett, J. M., "Assessment of commercial hemicellulases for saccharification of alkaline pretreated perennial biomass," Bioresource Technology, 102:1389-1398, (2011).

[11] Karagöz, P., "Lignocellulosic ethanol production via co-fermentation: Examining and enhancement of pretreatment and fermentation processes", PhD Thesis, Gebze Technology Institute, Kocaeli, Turkey (2013).

[12] Nigam, J.N., "Bioconversion of water-hyacinth (*Eichhornia crassipes*) hemicellulose acid hydrolysate to motor fuel ethanol by xylose-fermenting yeast", Journal of Biotechnology, 97(2):107–116, (2002).

[13] Zhao, X., Xiong, L., Zhang, M., Fengwu, B., "Towards efficient bioethanol production from agricultural and forestry residues: Exploration of unique natural microorganisms in combination with advanced strain engineering", Bioresource Technology, 215:84–91, (2016).

[14] Li, F., Ren, S., Zhang, W., Xu, Z., Xie, G., Chen, Y., Tu, Y., Li, Q., Zhou, S., Li, Y., Tu, F., Liu, L., Wang, Y., Jiang, J., Qin, J., Li, S., Li, Q., Jing, H.C., Zhou, F., Gutterson, N., Peng, L., "Arabinose substitution degree in xylan positively affects lignocellulose enzymatic digestibility after various NaOH/H_2SO_4 pretreatments in Miscanthus", Bioresource Technology, 130:629-637, (2013).

[15] Verardi, A, De Bari, I., Ricca, E., Calabrò, V., "Hydrolysis of lignocellulosic biomass: current status of processes and technologies and future perspectives", Bioethanol, 95–122, (2012).

[16] Xu, J., Cheng, J.J., Sharma-Shivappa, R.R., Burns, J.C., "Sodium hydroxide pretreatment of switchgrass for ethanol production", Energy Fuels, 24:2113–2119, (2010).

[17] Li, J., Zhou, P., Liu, H., Wu, K., Kang, X., Gong, Y., Xiao, W., Lin, J., Liu, Z., "A comparison of fermentation strategies for cellulosic ethanol production from NaOH-soaked sugarcane bagasse at high solid loading with decreased cellulase loading", Industrial Crops and Products, 62:446–452, (2014).

[18] Delgenes, J.P., Moletta, R., Navarro, J.M., "Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*", Enzyme and Microbial Technology, 19(3):220-225, (1996).

[19] Richard, P., Verho, R., Putkonen, M., Londesborough, J., and Penttilä, M., "Production of ethanol from L-arabinose by *Saccharomyces cerevisiae* containing a fungal L-arabinose pathway", FEMS Yeast Research, 3(2):185-189, (2003).

[20] Becker, J. and Boles, E., "A modified *Saccharomyces cerevisiae* strain that consumes L-arabinose and produces ethanol," Applied and Environmental Microbiology, 69(7):4144-4150, (2003).

[21] Akinosho, H., Yee, K., Close, D., Ragauskas, A., "The emergence of *Clostridium thermocellum* as a high utility candidate for consolidated bioprocessing applications", Frontiers in Chemistry, 2:66, (2014).

[22] Lynd, L.R., Guss, A.M., Himmel, M.E., Beri, D., Herring, C., Holwerda, E.K., Murphy, S.J., Olson, D.G., Paye, J., Rydzak, T., Shao, X., Tian, L., Worthen, R., "Advances in consolidated bioprocessing using *Clostridium thermocellum* and *Thermoanaerobacter saccharolyticum*", Industrial Biotechnology: Microorganisms, Volume 1, Chapter 10, Book Editor(s): Christoph Wittmann, James C. Liao, https://doi.org/10.1002/9783527807796.ch10, (2016).