

Article

# Comparison and Optimization of Saccharification Conditions of Alkaline Pre-Treated Triticale Straw for Acid and Enzymatic Hydrolysis Followed by Ethanol Fermentation

Rafał Łukajtis \*, Karolina Kucharska, Iwona Hołowacz, Piotr Rybarczyk, Katarzyna Wychodnik, Edyta Słupek, Paulina Nowak and Marian Kamiński

Faculty of Chemistry, Department of Chemical and Process Engineering, Gdańsk University of Technology, Narutowicza 11/12 Street, 80-233 Gdańsk, Poland; karolina.kucharska@pg.edu.pl (K.K.); iwona.holowacz@pg.edu.pl (I.H.); piotr.rybarczyk@pg.edu.pl (P.R.); katarzyna.wychodnik@pg.edu.pl (K.W.); edyta.slupek@pg.edu.pl (E.S.); paulina.nowak@pg.edu.pl (P.N.); marian.kaminski@pg.edu.pl (M.K.)

\* Correspondence: rafal.lukajtis@pg.edu.pl; Tel.: +48-58-347-23-10

Received: 19 February 2018; Accepted: 8 March 2018; Published: 13 March 2018

**Abstract:** This paper concerns the comparison of the efficiency of two-stage hydrolysis processes, i.e., alkaline pre-treatment and acid hydrolysis, as well as alkaline pre-treatment followed by enzymatic hydrolysis, carried out in order to obtain reducing sugars from triticale straw. For each of the analyzed systems, the optimization of the processing conditions was carried out with respect to the glucose yield. For the alkaline pre-treatment, an optimal catalyst concentration was selected for constant values of temperature and pre-treatment time. For enzymatic hydrolysis, optimal process time and concentration of the enzyme preparation were determined. For the acidic hydrolysis, performed with 85% phosphoric acid, the optimum temperature and hydrolysis time were determined. In the hydrolysates obtained after the two-stage treatment, the concentration of reducing sugars was determined using HPLC. The obtained hydrolysates were subjected to ethanol fermentation. The concentrations of fermentation inhibitors are given and their effects on the alcoholic fermentation efficiency are discussed.

**Keywords:** lignocellulosic biomass; enzymatic hydrolysis; acid hydrolysis; alkaline pre-treatment; optimization; Box-Behnken design; bioethanol fermentation

## 1. Introduction

Fermentation is an anaerobic respiration process carried out by many types of bacteria and fungi. Fermentation, by definition, is a disproportionation reaction. Ethanol fermentation is carried out by yeasts, including *Saccharomyces cerevisiae* [1–5]. Yeasts are able to ferment hexoses and some oligosaccharides, e.g., sucrose, maltose, or inulin, at specific conditions. A decreased pH value or the presence of inhibiting fermentation products, i.e., ethanol, influences the growthabilities of *Saccharomyces cerevisiae*. At ethanol concentrations up to 120 g/L, *Saccharomyces* are capable of performing fermentation and they are able to grow at ethanol concentrations up to 200 g/L [6–8].

The current global demand for biofuels is largely covered by the production of first-generation bioethanol from sucrose or starch contained in such raw materials as sugar cane, wheat, or corn. An interesting alternative is the production of the second-generation bioethanol as a result of fermentation processes from lignocellulosic waste. Lignocellulose, the most abundant renewable carbohydrate-rich biomass, is accumulated in large quantities from agricultural, forestry, or municipal activities and do not compete with food crops. It is also less expensive than conventional agricultural feedstocks. However, according to the complexity of the chemical structure, lignocellulosic biomass is

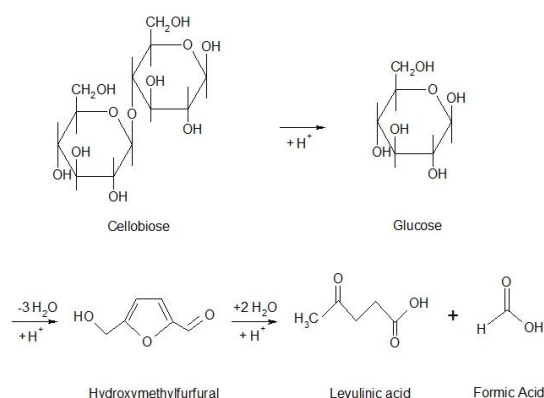
resistant to biodegradation. Cellulose, hemicellulose, and lignin, main components of lignocellulosic complex, are strongly intermeshed and bonded through covalent and non-covalent bonds. Lignin is the major barrier to make the carbohydrate components (cellulose and hemicellulose) suitable for bioconversion [9,10]. Therefore, the pre-treatment step has a large impact on subsequent enzymatic hydrolysis and fermentation steps [11]. It should improve the formation of sugars, be effective in the removal of lignin, and produce no, or limited amounts of, products that inhibit the action of the hydrolytic enzymes or the growth of fermentative microorganisms. Pre-treatment methods can be classified as biological, physical, chemical, and physico-chemical processes. A combination of different pre-treatment methods have also been considered [12].

The main goal of alkaline treatment, one of the most common chemical pre-treatment methods, is to remove lignin from the biomass [13]. The action of alkaline reagents leads to the degradation of side chains of esters and glycosides and then to the structural modification of lignin, cellulose swelling, cellulose decrystallization, and hemicellulose solvation.  $\text{Ca}(\text{OH})_2$  also removes acetyl groups from hemicellulose, reducing steric hindrance and increasing the availability of the enzyme to the surface of hemicellulose and cellulose. These methods are more effective for cellulose digestibility and for lignin solubilization, exhibiting minor cellulose and hemicellulose dissolution than acid or hydrothermal processes [9]. The advantage of alkaline methods is their low cost, especially when using  $\text{Ca}(\text{OH})_2$ . The limitation is that alkali are converted into un-salted or introduced as biomass salts after the pre-treatment and, therefore, the water consumption for salt removal from biomass is high. The lignin removal step is necessary to avoid the formation of inhibitors (salts, furfural, phenolic acid, aldehydes). Abudi et al. [14] investigated the effects of alkaline and peroxide pre-treatment of rice straw (RS). The results indicate that  $\text{NaOH}$  and  $\text{H}_2\text{O}_2$  pre-treatments affected the lignocellulose composition differently and are particularly effective in terms of solubilization of RS hemicellulose more than of cellulose and lignin.  $\text{NaOH}$  pre-treatment led to approximately 11%, 32%, and 22% reductions in hemicellulose, cellulose, and lignin content, respectively. In the case of  $\text{H}_2\text{O}_2$  pre-treatment, the corresponding reductions were 16%, 41%, and 7%, respectively.

After the pre-treatment of lignocellulosic biomass, the hydrolysates are further processed by biochemical methods. During the enzymatic hydrolysis, crystalline cellulose and hemicellulose not decomposed during pretreatment, and are degraded to reducing sugars, fermentable by microorganisms. The conversion of cellulose and hemicellulose is catalysed by cellulases and hemicellulases, respectively. Cellulases are usually a mixture of several enzymes. The three predominant ones are: endo-1,4- $\beta$ -glucanase, which hydrolyzes the inner  $\beta$ -1,4-glycosidic bonds; exo-1,4- $\beta$ -glucanase, which removes mono- and dimers from the free chain-ends; and  $\beta$ -glucosidase (cellobiase), which hydrolyzes glucose dimers [15–17]. Cellulose hydrolysis starts with adsorption of cellulase enzymes onto the surface of cellulose. Afterwards, cellulose is biodegraded to the fermentable sugars and cellulase is desorbed from the biomass surface. Cellulose microfibrils are surrounded by hemicellulose polysaccharides. Therefore, auxiliary enzymes that attack hemicellulose are used. In the first step, endo-1,4- $\beta$ -xylanase depolymerizes xylan into xylooligosaccharides. Further, xylanases, such as  $\beta$ -glucuronidase,  $\alpha$ -arabinofuranosidase, and acetyl xylan esterase, cleave side chains and side groups of heteroxylan, while galactomannanase and glucomannanase hydrolyze glucomannan [18]. The optimal balanced combination of enzymes is needed to effectively modify the complex structure of lignocellulosic materials. In addition to the quality of the hydrolyzing enzymes, the digestibility of cellulose and hemicellulose is affected by pH, temperature, process time and porosity, degree of crystallization, and cellulose content [19].

Saccharification of lignocellulosic biomass with an acid catalyst is also possible. The main objective of acid treatment is the solubilization of hemicellulose and making cellulose accessible to enzymes. Acid hydrolysis is an alternative for enzymatic hydrolysis, as acid hydrolyzes biomass to fermentable sugars. However, intensive washing is required to remove the acid before fermentation is performed. The advantage of using acids is their low price and availability. Additionally, the use of organic acids (formic, acetic, maleic) makes the process environmentally friendly [20]. The disadvantage is the

need to neutralize the used acid by alkali, which generates a stream of reagents, or by vacuum distillation, in order to reuse the acid. Another disadvantage of using acid hydrolysis is the possibility of the formation of fermentation inhibitors, especially when high acid concentrations, high temperatures, and long reaction times are applied [21,22]. During acid hydrolysis, the acid catalyzes the breakdown of glycosidic bonds between sugar residues, followed by the breakdown of released glucose to form hydroxymethylfurfural (HMF), formic acid (FA), and levulinic acid (LA) (Figure 1) [23]. Compounds, such as furfural, 5-HMF, and phenolic compounds affect the microorganism metabolism in the fermentation step. Dilute acid pre-treatment is more favourable for industrial applications due to the reduction of the operational and maintenance costs (corrosion problems). Additionally, this kind of pre-treatment generates fewer degradation compounds than the concentrated acid pre-treatment [18,24,25].



**Figure 1.** Formation of selected fermentation inhibitors from glucose during acid hydrolysis.

The lignocellulosic hydrolysates are very attractive as a substrate for fermentative conversion processes to bioethanol. The results of the latest research on the production of bioethanol from waste lignocellulosic biomass are presented in Table 1.

**Table 1.** The efficiency of bioethanol production related to the pre-treatment method and used microorganisms.

Type of Lignocellulosic Material	Type of Pretreatment	Used Microorganism	Ethanol Yield (g/g)	Processes Involved	References
Cotton residues, corn stover	-	<i>Saccharomyces cerevisiae</i>	0.027	Hydrolysis and fermentation realized separately	[21]
Wheat straw	Steam explosion	<i>Kluyveromyces marxianus</i>	0.27	Simultaneous saccharification and fermentation	[26]
Sugarcane bagasse	Acid digestion, steam explosion	<i>Pichia stipitis</i>	0.39	Simultaneous saccharification and fermentation	[27]
Sugarcane bagasse	Milling	<i>Pichia stipitis</i>	0.29	Simultaneous saccharification and fermentation	[28]
			0.27	Hydrolysis and fermentation realized separately	
Rice husks	Alkaline digestion	<i>Escherichia coli</i>	0.21	Hydrolysis and fermentation realized separately	[29]
			0.20	Simultaneous saccharification and fermentation	

Table 1. Cont.

Type of Lignocellulosic Material	Type of Pretreatment	Used Microorganism	Ethanol Yield (g/g)	Processes Involved	References
Maize cob	Acid digestion, overliming	<i>Zymomonas mobilis</i>	0.42	Batch reactor	[30]
	Drying	<i>Clostridium thermocellum</i> ;	0.36		
	Acid digestion	<i>Clostridium</i>	0.40		
Banana remnants	Alkaline digestion	<i>thermosaccharolyticum</i>	0.42	Batch reactor	[31]
	Drying	<i>Clostridium</i>	0.33		
	Acid digestion	<i>thermocellum</i> ;	0.36		
	Alkaline digestion	<i>Clostridium ethanolicus</i>	0.39		

Currently, lignocellulosic biomass is regarded as the forward-looking, large-scale available non-fossil carbon source for biofuels production. Therefore, there is a strong motivation for research into industrially and economically viable processes for conversion of biomass to fuels, including bioethanol. However, successful and effective conversion of lignocellulosic biomass to monosaccharides in high yields is still a challenge. Acid or enzymatic hydrolysis of biomass polysaccharides after a pre-treatment stage are the main currently-investigated saccharification methods [32–36]. Therefore, the primary objective of the present study is the comparison of the efficiency of two-stage hydrolysis processes, i.e., alkaline pre-treatment followed by acid hydrolysis, as well as alkaline pre-treatment followed by enzymatic hydrolysis, carried out in order to obtain reducing sugars from triticale straw. The comparison is to concern not only results obtained for mono sugars, but for bioethanol as well.

The aim of the work is also to examine the course and efficiency of the enzymatic hydrolysis using cellulolytic enzymes immobilized on diatomite. This procedure is intended to reduce process costs through the possibility of enzyme regeneration and reuse.

The optimization of the conditions of the two-stage hydrolysis and the evaluation of the influence of process parameters on the glucose yield were performed according to the Box-Behnken design for the following variables: concentration of NaOH, duration time of acid hydrolysis, temperature of acid hydrolysis, the content of Viscozyme L, and the duration time of the enzymatic hydrolysis process. Finally, the parameters' significance on the performance of sugars were optimized using the response surface methodology. The effect of acid hydrolysis conditions on the content of typical fermentation inhibitors, including 5-HMF, furfural, and the total phenolic compounds was investigated. Additionally, pre-treatment and hydrolysis effects were evaluated by performing bioethanol fermentation on previously-obtained hydrolysates.

## 2. Results and Discussion

### 2.1. Biomass Characterisation

Milled and minced lignocellulosic biomass (triticale straw *Triticum* sp., originated from Wejherowo, Poland) was used in this study. The determined composition of the *Triticum* sp. was found to be: 43.6% cellulose, 23% hemicellulose, 21.5% lignin, 8.3% water extractives, 8.6% ethanol extractives, 1.2% ash, and a moisture content of 6.2% (on dry weight basis). Hemicellulose content was found as follows: xylose (18.1%), galactose (4.1%), and mannose with arabinose (0.8%).

### 2.2. Influence of Alkaline Pre-Treatment Conditions of the Chemical Composition of Triticale Straw

The optimization of the two-stage hydrolysis conditions and the evaluation of the influence of process parameters on the glucose yield were made for two test systems. In the first system, alkaline pre-treatment and acid hydrolysis were carried out, while, in the second system, alkaline pre-treatment

followed by enzymatic hydrolysis. In both cases, the alkaline pre-treatment was carried out for six hours at a constant temperature of 65 °C. The treatment was carried out for the catalyst concentrations (NaOH) of 1%, 6%, and 11%. In the first stage of the research, the effect of alkaline pre-treatment on the change of triticale straw composition was determined. For this purpose, the composition of biomass (reducing sugars and lignin contents), as well as the degree of lignin removal, the degree of cellulose and hemicellulose recoveries were determined in the residue remaining after the alkaline treatment. The results (Table 2) indicate that an increase in the concentration of NaOH causes an increase in the biomass loss of 37%, 49%, and 52%, respectively, for catalyst concentrations of 1%, 6%, and 11%. A similar dependence was obtained in [12,37,38]. The research results indicate that the use of 1% NaOH already significantly lowers the lignin content. A further increase in the catalyst concentration above 1% does not significantly affect the lignin removal. The alkaline pre-treatment of the material influences the change in the proportion of the sugar polymer content. The use of 1% NaOH allows the content of all reducing sugars to increase. For NaOH concentrations greater than 1%, a decrease in reducing sugars coming from hemicellulose with a simultaneous increase in glucose concentration is observed.

**Table 2.** Composition of raw biomass and obtained hydrolysates.

	Glucose Content (mg/g <sub>biomass</sub> )	Xylose Content (mg/g <sub>biomass</sub> )	Galactose Content (mg/g <sub>biomass</sub> )	Mannose, Arabinose Content (mg/g <sub>biomass</sub> )	Lignin Content (%)	Cellulose Recovery (%)	Hemicellulose Recovery (%)	Lignin Removal (%)
Untreated	436	181	41	8	21.5	-	-	-
1% NaOH	548	230	58	13	9.5	79.3	82.4	72.1
6% NaOH	644	171	43	0	8.4	75.4	47.4	75.7
11% NaOH	701	141	22	0	8.4	77.2	33.9	75.2

### 2.3. Optimisation of Alkaline Pre-Treatment Followed by Acid Hydrolysis

For the analysis of the influence of input variables on the result of two-stage hydrolysis, i.e., alkaline treatment and acid hydrolysis, experiments (Table 3) were performed according to the Box-Behnken design for three variables. The following boundary values were assumed for the investigated variables: concentration of NaOH 1–11% at a constant temperature of 65 °C and alkaline pre-treatment time of 6h, duration of acid hydrolysis of 0.5–8 h, and the temperature of acid hydrolysis of 60–120 °C. As an output parameter, glucose yield ( $G_y$ ) was selected as a parameter describing the efficiency of the process. Additionally, Table 3 shows the obtained values of the sum of reducing sugars.

**Table 3.** Box-Behnken design for alkaline pre-treatment and acid hydrolysis of *Triticum* sp.

No.	C <sub>NaOH</sub> (%)	Temperature (°C)	Time (h)	$G_y$ (mg/g <sub>biomass</sub> )	Sum of Sugars (mg/g <sub>biomass</sub> )
1	1	90	0.5	334.5	397.3
2	11	90	0.5	293.9	360.8
3	1	90	8	104.6	122.3
4	11	90	8	106.4	118.5
5	1	60	4.25	229.6	314.6
6	11	60	4.25	204.9	285.0
7	1	120	4.25	7.3	13.2
8	11	120	4.25	7.6	8.2
9	6	60	0.5	56.4	147.9
10	6	60	8	301.7	369.3
11	6	120	0.5	271.4	325.3
12	6	120	8	14.3	14.3
13	6	90	4.25	265.6	314.8
14	6	90	4.25	260.7	312.8
15	6	90	4.25	269.8	326.1

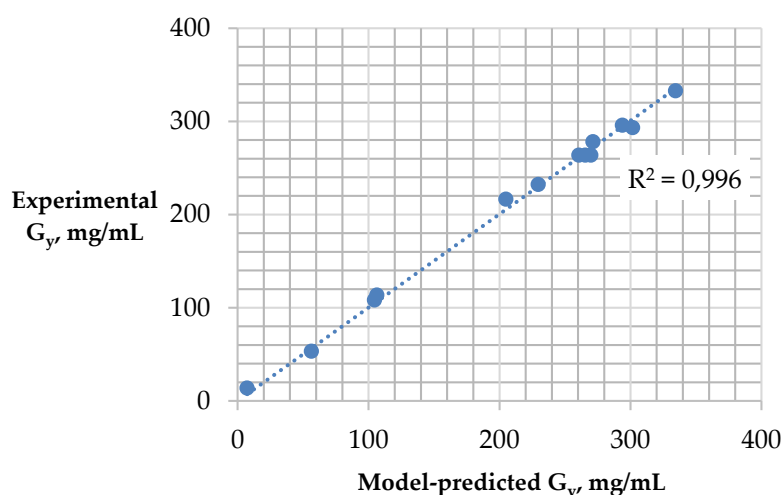
An influence of the process parameters (NaOH concentration, temperature and duration of acid hydrolysis) on the glucose yield was investigated. On the basis of multiple regression, the Equation (1) was proposed, describing the relationships occurring during the two-stage hydrolysis.

$$Y [\%] = -1426 - 3.98X_1 + 34.45X_2 + 177.70X_3 - 0.16X_2^2 - 3.64X_3^2 + 0.57X_1 \cdot X_3 - 1.97X_2 \cdot X_3 \quad (1)$$

In Equation (1),  $X_1$ ,  $X_2$ , and  $X_3$  represent sodium hydroxide concentration, temperature, and time of acid hydrolysis, respectively.  $Y$  denotes glucose efficiency. Equation (1) enables calculating the theoretical glucose yield within the range of variables according to the boundary conditions. The parameters of Equation (1) are given in Table 4. In order to determine the effect of the parameters significance on the performance of sugars, an analysis of variance for the surface of the model's response was made. The form of Equation (1) is the result of rejecting the least significant parameters in the next iterative steps. This procedure showed that the least important parameters in such a system is the interaction between concentration of catalyst and temperature ( $X_1 \cdot X_2$ ). For this interaction, the  $p$ -value was much higher than the assumed significance level  $p < 0.1$ , which is why it was rejected. Based on analysis of variance, the obtained value of the determination coefficient ( $R^2 = 0.996$ , Figure 2) indicates a very good correlation between the efficiency calculated on the basis of Equation (1) and the actual glucose efficiency within the investigated range of variables.

**Table 4.** Model parameters for Box-Behnken design for alkaline pre-treatment and acid hydrolysis of *Triticum* sp.

Parameter	Value	Std. Error	$t$ -Value	$p$ -Value
Intercept	-1426	53.340	-26.746	$1.81 \times 10^{-7}$
$X_1$	-3.98	1.234	-3.227	0.0180
$X_2$	34.45	1.118	30.824	$7.74 \times 10^{-8}$
$X_3$	177.70	6.485	27.397	$1.56 \times 10^{-7}$
$X_2^2$	-0.16	0.006	-28.087	$1.35 \times 10^{-7}$
$X_3^2$	-3.64	0.376	-9.663	$7.04 \times 10^{-5}$
$X_1 \cdot X_3$	0.57	0.246	2.295	0.0615
$X_2 \cdot X_3$	-1.97	0.055	-35.631	$3.26 \times 10^{-8}$

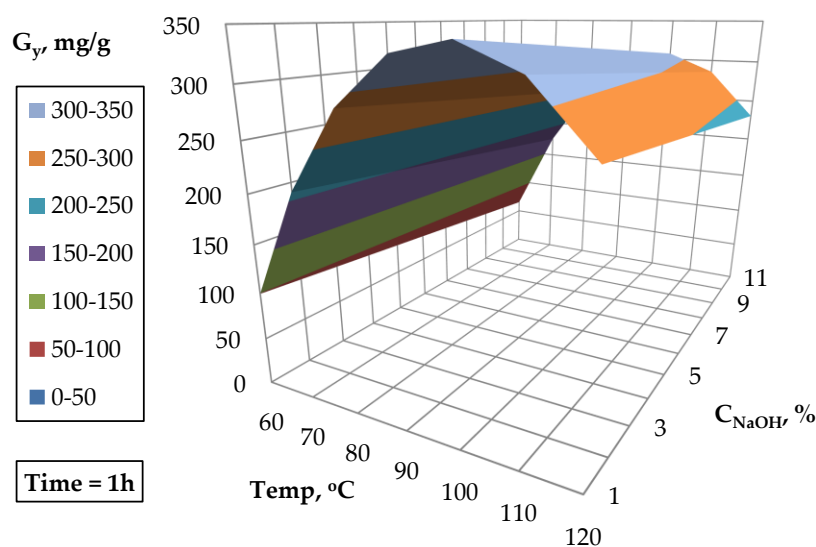


**Figure 2.** Analysis of variance for the theoretical (model-predicted) and experimental glucose yield for alkaline pre-treatment and acid hydrolysis.

The attached figures show model surface responses and are the effect of the interactions between two selected variable parameters, while maintaining the third parameter at a constant level. The model response is the concentration of glucose (glucose yield) after the two-stage hydrolysis, where in the

second stage the obtained solid fraction after the alkaline pre-treatment was used. The analysis of the surface response was useful for the determination of the individual effects of each parameter and the impact of interactive effects of independent variables on the glucose yield. This approach allowed to determine the optimal conditions with respect to each investigated parameter.

Figure 3 presents the effect of NaOH concentration and acid hydrolysis temperature on glucose yield (acid hydrolysis time 1 h). The results indicate that the glucose yield reaches a maximum at about 100 °C. Increasing the temperature of acid hydrolysis above 100 °C results in a decrease of glucose yield due to the breakdown of simple sugars. The increase in NaOH concentration during the pre-treatment causes a decrease in a glucose yield. This is caused by a significant loss of mass during alkaline pre-treatment. Biomass loss is related to the catalyst concentration and it is equal to 38% for the treatment of 1% NaOH and 52% when 11% NaOH is used.



**Figure 3.** The surface response plots of the effects of NaOH concentration and temperature acid hydrolysis on the glucose yield.

Figure 4 shows the effect of temperature and duration time of acid hydrolysis on the glucose yield for a constant sodium base concentration (6%). Glucose efficiency increases with increasing temperature, however, when the optimal temperature (100 °C) is reached, a significant decrease in glucose efficiency is observed if the temperature is further increased. For temperatures in the range of 90–100 °C, and for a short time of acid hydrolysis (0.5 h), the highest glucose yields are obtained. An increase in time over 0.5 h results in a decrease in the hydrolysis efficiency. Only for the temperature of 60 °C, an increase in glucose efficiency along with the duration time of acid hydrolysis is observed.

Figure 5a,b present an influence of the duration time of acid hydrolysis and NaOH concentration (alkaline treatment) on the glucose yield at a constant temperature of acidic hydrolysis. Two possibilities of interperation of two-stage hydrolysis should be considered here. In one variant, when the acid hydrolysis temperature is 100 °C (Figure 5b), high yield of reducing sugars can only be achieved for a short duration of acidic hydrolysis (0.5 h). The increase in the duration of treatment results in a decrease of the glucose yield due to the degradation of simple sugars. When a lower temperature of 60 °C is applied (other variant, Figure 5a), the highest yield is achieved after 8 h of acidic hydrolysis. The choice of process temperature, besides the efficiency of reducing sugars, should also be supported by the concentration of fermentation inhibitors in the resulting hydrolysate.

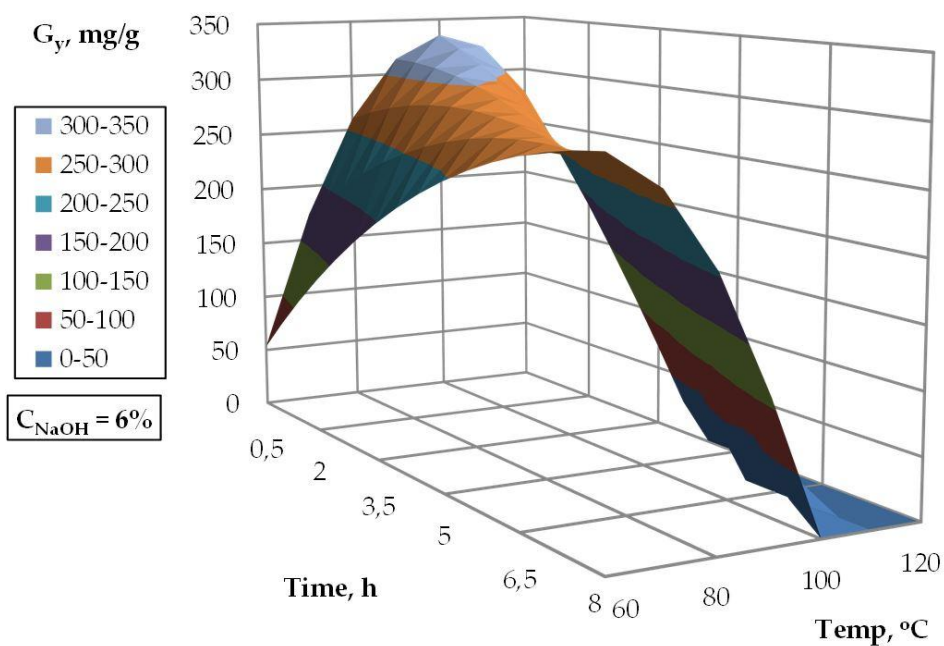


Figure 4. The surface response plots of the effects of temperature and time on the glucose yield.

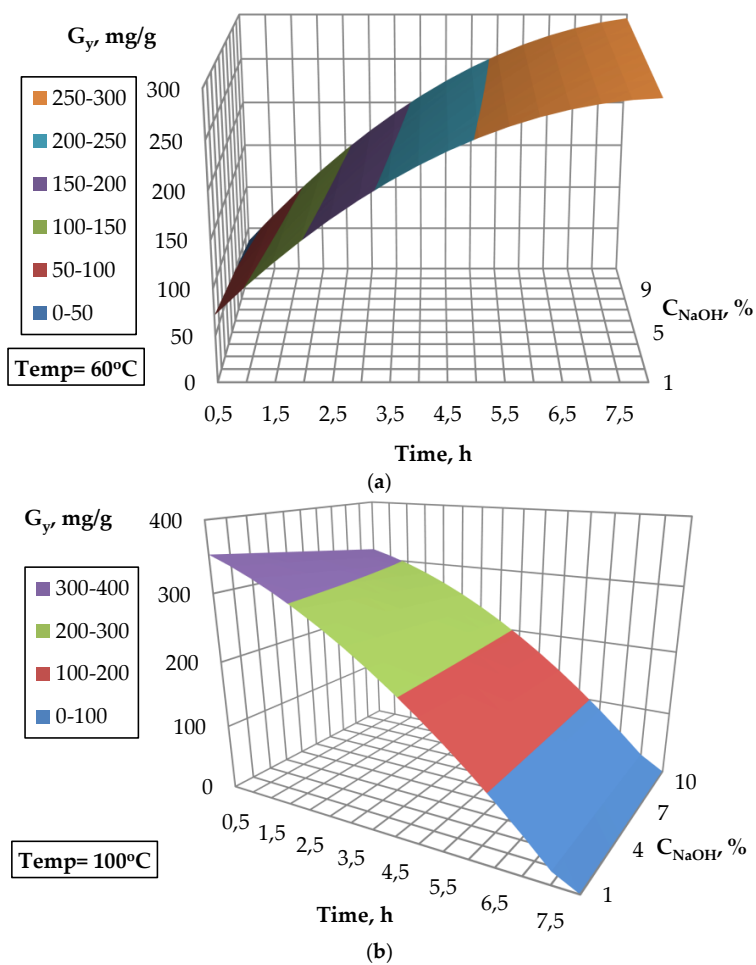


Figure 5. The surface response plots of the effects of NaOH concentration and time acid hydrolysis on the glucose yield: (a) at a constant temperature 60 °C; (b) at a constant temperature 100 °C.



The analysis of the surface response areas (Figures 3–5) allowed to determine the optimal conditions for a two-stage process involving alkaline pre-treatment (6% NaOH, 6 h, 65 °C) and acid hydrolysis (100 °C, 0.5 h and 85% H<sub>3</sub>PO<sub>4</sub>). For these conditions, hydrolysis of the triticale straw was performed to obtain, from 1 g of raw material, 320 mg of glucose, 42.8 mg of xylose, 19 mg of galactose and 1.5 mg of arabinose, respectively. A similar result of the yield of sugars was obtained for acid hydrolysis with sulfuric acid (70% H<sub>2</sub>SO<sub>4</sub>, 30 °C, 2 h) of *Saccharum spontaneum*, also after treatment with sodium base (7%, 48 h, 30 °C). For these conditions, a maximum yield of reducing sugars was obtained 369.6 mg/g [14]. The acid hydrolysis was carried out on straw which had not been subjected to the previous alkaline pre-treatment, obtaining, from 1 g of raw material, 420 mg of glucose, 150 mg of xylose, 38 mg of galactose and 4.7 mg of arabinose, respectively. It is clear that the efficiency of reducing sugars for acid hydrolysis with the omission of the alkaline pre-treatment is the highest.

#### 2.4. Optimisation of Alkaline Pre-Treatment Followed by Enzymatic Hydrolysis

The system consists of 15 experiments planned according to the Box-Behnken design. Experiments include two-stage hydrolysis of biomass, i.e., alkaline pre-treatment followed by enzymatic hydrolysis. During the alkaline pre-treatment, NaOH concentration in the first step of hydrolysis is optimized at a constant process temperature of 65 °C, pre-treatment time of 6h, and granulation of 0.75 mm. In the second step, enzymatic hydrolysis values of the time and ratio of cellulolytic enzymes immobilized on a solid support are optimized. As the boundary conditions, on the basis of previous experiments, NaOH concentrations of 1%, 6%, and 11% were assumed, and for enzymatic hydrolysis time 8, 16, and 24 h and the content of Viscozyme L in a mixture of 0.9, 0.95, and 1 were selected. Box-Behnken design estimation allowed the determination of the response surface based on the quadratic equation. As the initial parameter representing the efficiency of the process, the mass of the obtained glucose was selected (glucose yield, G<sub>y</sub>). Table 5 shows the obtained values of the glucose and the sum of reducing sugars.

**Table 5.** Box-Behnken design for alkaline pre-treatment and enzymatic hydrolysis of *Triticum* sp.

No.	C <sub>NaOH</sub> (%)	Viscozyme L	Time (h)	G <sub>y</sub> (mg/g <sub>biomass</sub> )	Sum of Sugars (mg/g <sub>biomass</sub> )
1	1	0.90	16	234.7	278.8
2	11	0.90	16	250.1	292.8
3	1	1.00	16	186.2	245.3
4	11	1.00	16	207.6	289.4
5	1	0.95	8	143.9	184.0
6	11	0.95	8	189.4	249.7
7	1	0.95	24	219.8	268.5
8	11	0.95	24	159.7	195.4
9	6	0.90	8	208.4	289.0
10	6	1.00	8	163.1	204.2
11	6	0.90	24	234.8	275.9
12	6	1.00	24	218.3	257.2
13	6	0.95	16	231.5	302.0
14	6	0.95	16	235.6	296.8
15	6	0.95	16	227.4	281.4

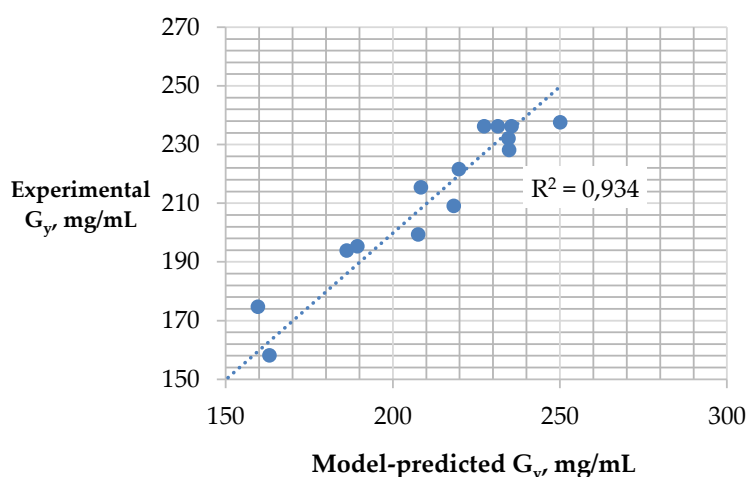
The influence of the input parameters, i.e., the NaOH concentration, the Viscozyme L content, and the duration time of the enzymatic hydrolysis process have been examined considering the glucose yield as the model output. The equation describing the relationships occurring during the two-stage hydrolysis was determined by multiple regression. The obtained regression equation describing the surface response takes the form:

$$Y [\%] = 700.18 - 20.89X_1 - 763.82X_4 - 0.82 X_1^2 - 0.52X_5^2 - 0.66X_1 \cdot X_5 + 23.86X_4 \cdot X_5 \quad (2)$$

In Equation (2)  $X_1$ ,  $X_4$ , and  $X_5$  represent sodium hydroxide concentration, Viscozyme L content, and the time of enzymatic hydrolysis, respectively.  $Y$  denotes a glucose yield. Equation (2) enables the calculation of the theoretical glucose yield within the range of variables according to boundary conditions. In order to obtain Equation (2), the least significant parameters were rejected in the consecutive iterative steps. It turned out that the least important parameters are: NaOH concentration, duration of enzymatic hydrolysis, and the ratio of enzymes raised to the square. Equation 2 is described by the model parameters contained in Table 6. Based on the analysis of variance, the obtained value of the determination coefficient ( $R^2 = 0.934$ ) indicates a good correlation of the results calculated from the equation with the experimental results of glucose efficiency (Figure 6).

**Table 6.** Model parameters for the Box-Behnken design for alkaline pre-treatment and enzymatic hydrolysis of *Triticum* sp.

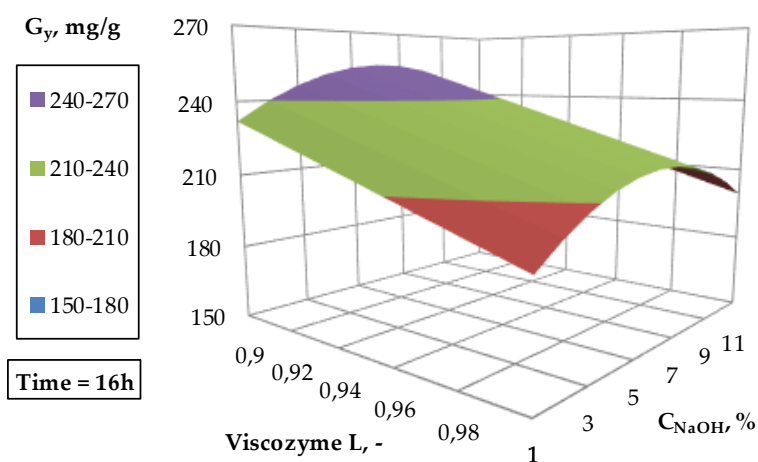
Parameter	Value	Std. Error	t-Value	p-Value
Intercept	700.177	75.480	9.276	$1.48 \times 10^{-5}$
$X_1$	20.887	3.463	6.031	0.000312
$X_4$	-763.822	88.777	-8.604	$2.58 \times 10^{-5}$
$X_1^2$	-0.819	0.220	-3.725	0.005830
$X_5^2$	-0.523	0.084	-6.233	0.000250
$X_1 \cdot X_5$	-0.656	0.132	-4.964	0.001102
$X_4 \cdot X_5$	23.864	2.975	8.022	$4.281 \times 10^{-5}$



**Figure 6.** Analysis of variance for the theoretical (model-predicted) and experimental glucose yield for alkaline pre-treatment and enzymatic hydrolysis.

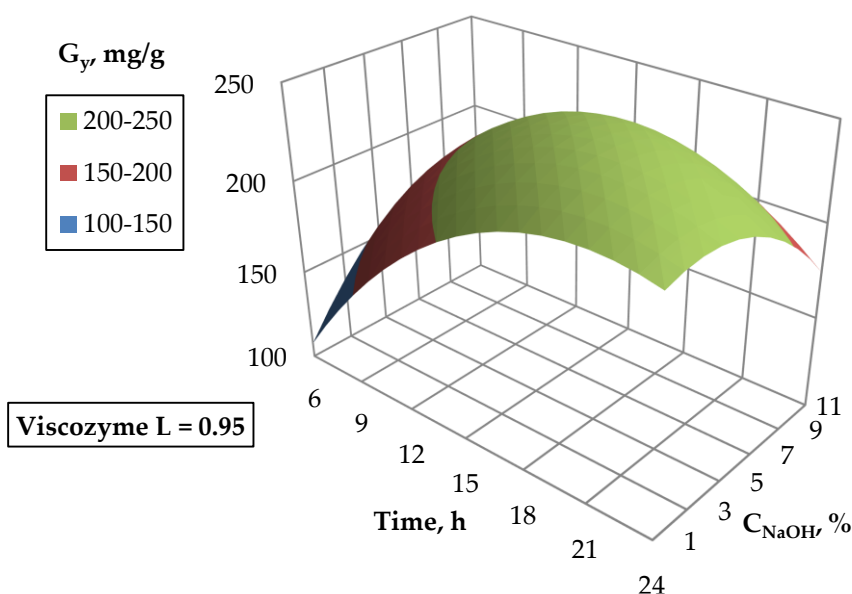
The analysis of reaction mixture samples shows that the main product of lignocellulosic biomass hydrolysis is mainly glucose. Table 5 presents the yield of this monosugars in the hydrolysates. The obtained result was compared with the sum of all reducing sugars. Physical studies have shown low content of cellobiose in hydrolysates, therefore, it was crucial to use a small addition of  $\beta$ -glucosidase in a mixture with Viscozyme L. Cellobiose is an inhibitor of cellulolytic enzymes and its presence lowers the efficiency of enzymatic hydrolysis. Figure 7 presents exemplary changes in glucose efficiency for enzymatic hydrolysis conducted for 16h in the range of variables described in Table 5. Based on the shape of the response surface it can be stated that for the change in NaOH concentration, a local maximum around 9% is observed, above and below which the obtained glucose yields are lower. Therefore, 9% NaOH concentration is chosen as the optimal value. Figure 7 also shows that there is a linear relationship between the yield of glucose and the ratio of  $\beta$ -glucosidase in the mixture with Viscozyme L during the enzymatic hydrolysis, where an increase in the ratio of  $\beta$ -glucosidase (or the decrease of Viscozyme L) corresponds with an increase in the yield of glucose after enzymatic hydrolysis.

The mentioned changes are small enough to decide that the optimal addition of  $\beta$ -glucosidase chosen for further experiments is 0.05 in proportion to Viscozyme L equal to 0.95.



**Figure 7.** The surface response plots of the effects of NaOH concentration and Viscozyme L content on the glucose yield after 16 h of enzymatic hydrolysis.

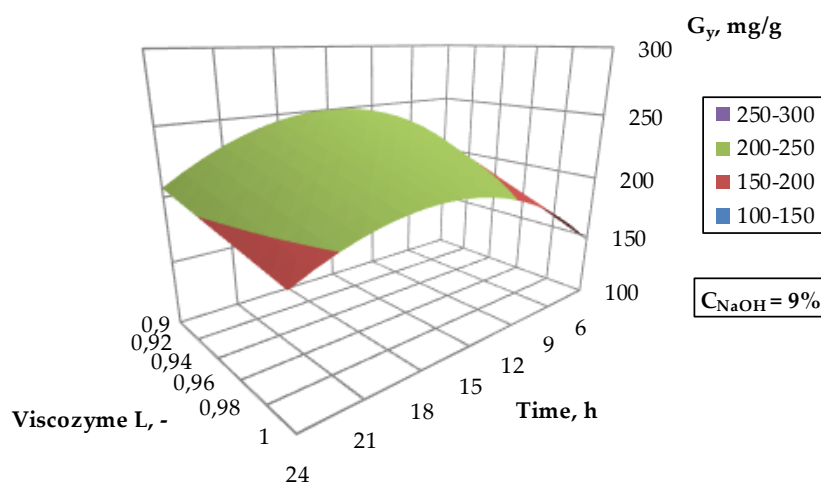
Figure 8 presents changes in glucose efficiency for enzymatic hydrolysis carried for Viscozyme L content of 0.95. Based on the course of the response surface it can be stated that for the change in NaOH concentration, a local maximum around 7–9% is observed. Obtained results confirm that 9% NaOH concentration is an adequate concentration of alkaline catalyst chosen as the optimal value. A relationship between time and glucose yield during enzymatic hydrolysis show that an increase of the hydrolysis time above 16 h is no longer reasonable. It was decided that the optimal time of enzymatic hydrolysis is equal to 16 h for Viscozyme L content of 0.95 and 9% concentration of NaOH.



**Figure 8.** The surface response plots of the effects of NaOH concentration and time on the glucose yield for enzyme ratio of 0.95.

Figure 9 presents changes in response surface for glucose efficiency during the two-step alkaline and enzymatic hydrolysis conducted for NaOH concentration equal to 9%. A maximum in the range of about 15–17 h of the enzymatic hydrolysis time can be observed. The ratio of  $\beta$ -glucosidase to

Viscozyme L does not show any significant changes in the hydrolysis course. Yet, the addition of  $\beta$ -glucosidase to Viscozyme L increases the cost of enzymatic hydrolysis, therefore, the content of Viscozyme L of 0.95 is taken as optimal on the basis of results given in Figures 7 and 9.



**Figure 9.** The surface response plots of the effects of Viscozyme L content and hydrolysis time on the glucose yield for NaOH concentration of 9%.

Based on statistical analysis of experimental data and the analysis of response surface course and changes optimal values for further experiments have been determined. It can be stated that a NaOH concentration of 9%, an enzyme ratio equal to 0.95, and a time of 16 h is the set of optimal conditions for two-step alkaline-enzymatic hydrolysis.

### 2.5. Influence of the Pre-Treatment and Saccharification Conditions on the Formation of Fermentation Inhibitors

For the hydrolysis experiments using phosphoric acid according to the Box-Benken design, the content of typical fermentation inhibitors including 5-HMF, furfural, and the total phenolic compounds was determined (Table 7). The application of acid hydrolysis involves the possibility of the transformation of sugars (including pentoses) to furfural and levulinic acid, and to 5-HMF in the case of glucose [23,39]. Partial hydrolysis of lignin as a result of which phenolic compounds pass into the hydrolysate solution is also possible. Therefore, the use of acid in the saccharification process of lignocellulosic biomass may be associated with the formation of compounds negatively affecting the metabolism of microorganisms (so-called inhibitors). Depending on the concentration, inhibitors may completely block the course of fermentation processes or affect the products of microbial metabolism [40,41]. Presence of inhibitors in the hydrolysate often involves the need to perform the detoxification prior to fermentation. In this work, the effect of the hydrolysis conditions with 85% phosphoric acid solution on the content of fermentation inhibitors was investigated. Analysis of the obtained results shows that temperature of hydrolysis is the most significant parameter influencing the formation of fermentation inhibitors. The rate and efficiency of catalytic conversion of simple sugars to furfural and HMF is higher for higher temperatures of the hydrolysis. At temperatures above 100 °C, degradation processes occur in which, apart from the destruction of sugars, there is also degradation of furfural, HMF, as well as phenolic compounds. The effects of degradation increase with the increase of the duration time of the hydrolysis process. This is why when the hydrolysis time is 8 h and the hydrolysis temperature is 120 °C, the concentrations of simple sugars and inhibitors are almost zero. The performance of the hydrolysis at lower temperature leads to the formation of inhibitors, however, their concentration is much lower than for the process carried out at higher temperature and for the same time. Comparing the processes carried out at temperatures of 90 and 60 °C for the duration time resulting in similar efficiency of reducing sugars (i.e., 0.5 h, 90 °C

and 8 h, 60 °C), the concentration of selected inhibitors is similar (experiments 1 and 10, Table 7). The concentration of total phenolic compounds is additionally affected by the alkaline pre-treatment. The use of alkaline treatment prior to acid hydrolysis (AlAcH) allowed to reduce the concentration of phenolic compounds by 29.2 mg/g<sub>biomass</sub>, and furfural by 27.5 mg/g<sub>biomass</sub> as compared to the process without alkaline treatment (AcH). However, the concentration of 5-HMF is almost doubled when alkaline pre-treatment is applied, mainly due to the higher content of glucan in the material after the alkaline pre-treatment. The higher the concentration of NaOH, the lower the concentrations of furfural and total phenolic compounds and the higher the concentration of NaOH, the higher the concentration of 5-HMF (compare the experiments i.e., 2 and 1, 4 and 3, etc., Table 7). No vanillin, vanillic acid, and guaiacol was identified in the investigated samples of hydrolysates. In order to determine the effect of inhibitors on the bioethanol yield, detoxification of AlAcH and AcH hydrolysates was carried out. For this purpose, sorption on silica gel modified with octadecyl groups was applied. The use of this type of adsorbent allowed the almost complete removal of furfural and phenolic compounds and about a three- to five-fold decrease in 5-HMF concentration. The applied method of detoxification is effective and allows for the removal of phenolic compounds and furan derivatives from the hydrolysates, without the simultaneous loss of reducing sugars. Authors of [42] applied a XAD-4 sorbent polymer. As a result of detoxification with this method, 5-HMF was completely removed and the furfural concentration was reduced by about 6.5 times. In addition, acetic acid was also removed by means of electro dialysis. Interestingly, commonly-used activated carbon does not allow for complete removal of fermentation inhibitors. For example, the use of a two-stage detoxification (combination of hydrolysis treatment with calcium hydroxide and activated carbon) allows to remove 78.3% of the total phenolic and furan compounds from the solution after two-stage hydrolysis with sulfuric acid [43]. The use of electro dialysis allows for the complete removal of acetic acid and more than 50% of total phenolic compounds, however, it has no significant effect on reducing the concentration of furan derivatives [44]. After enzymatic hydrolysis, as in this study, no phenolic compounds, 5-HMF, and furfural were identified in the hydrolysate [45].

Table 7. Determined inhibitors of fermentation.

Experiment	Total Phenolic Compounds (mg/g)	Furfural (mg/g)	5-HMF (mg/g)
1	44.0	73.6	7.2
2	29.0	42.1	8.9
3	17.7	2.9	3.3
4	16.0	2.1	4.3
5	17.9	24.3	2.6
6	17.4	16.6	4.3
7	6.4	0.0	0.0
8	4.8	0.0	0.0
9	9.4	2.8	0.6
10	32.7	45.0	8.8
11	49.3	53.8	16.9
12	4.8	0.0	0.0
13	19.9	10.4	8.1
AcH	76.1	67.4	16.5
AlAcH	46.9	39.9	18.8
AcHD	1.5	0.0	4.9
AlAcHD	0.0	0.0	3.2

## 2.6. Influence of the Pre-Treatment on the Bioethanol Yield

The industry uses various species and breeds of yeast belonging to the genus *Saccharomyces*. They are able to ferment simple sugars, i.e., glucose, fructose, mannose, maltose, and sucrose. During intensive fermentation, yeast cells are distributed throughout the entire volume of fluid. The yeasts of the upper fermentation (*Saccharomyces cerevisiae*) conduct the process at a higher

temperature than the yeast of the lower fermentation (*Saccharomyces carlsbergensis*), the process itself is more intensive with the release of a large amount of CO<sub>2</sub>.

In this work, small-scale alcoholic fermentation was carried out. Hydrolysates of triticale straw, obtained as a result of alkaline pre-treatment and acid or enzymatic hydrolysis, carried out under previously-optimized conditions, were used as the process feed. Acid hydrolysis with 85% phosphoric acid (hydrolysis time of 0.5 h and temperature of 100 °C) was performed for untreated straw (AcH), as well as for the alkaline pre-treated straw (AlAcH, 6% NaOH, 6 h, 65 °C). The enzymatic hydrolysis under optimal conditions (EH, hydrolysis time of 16h, Viscozyme L content of 0.9, hydrolysis temperature of 42 °C) was also performed for the untreated material and for the alkaline pre-treated material (AlEH). In addition, acidic hydrolysates were detoxified (AcHD and AlAcHD, respectively). The results were compared to the results of fermentation carried out on glucose, untreated straw, and alkaline pre-treated straw. The course and results obtained for the different types of treatment were evaluated. Each time, the result refers to the amount of sludge or hydrolysate obtained from 3 g of raw material. The differences in the amount of the pre-treatment method on the sugar composition of hydrolysates, as well as the composition of the broth after the fermentation processes are presented in Table 8.

The results indicate that the yeasts *Saccharomyces cerevisiae* are unable to digest sugar polymers present in the plant material if no cellulolytic enzymes are present in the reaction mixture. Figure 10 presents the courses of the fermentation processes. The highest ethanol yield was obtained after 9 h of the fermentation processes.

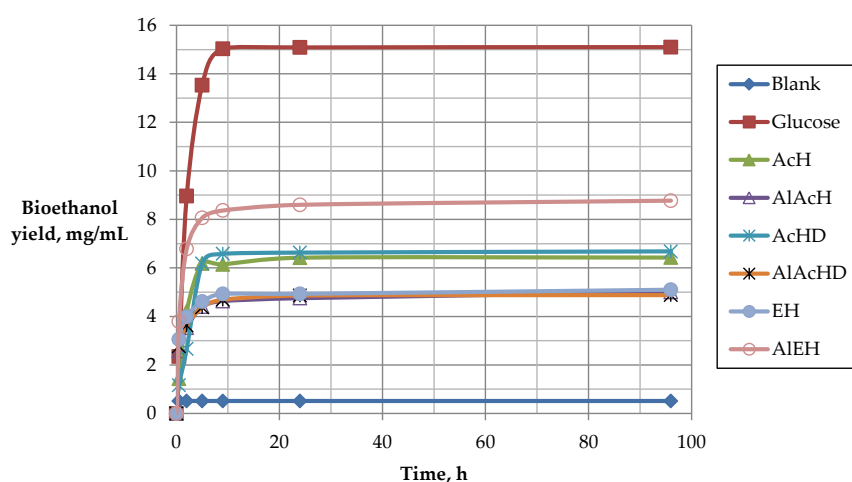


Figure 10. Courses of performed fermentations.

The highest ethanol concentration (8.8 mg/mL), corresponding to a yield of 0.29 g/g<sub>biomass</sub>, was obtained for the fermentation carried out for alkaline pre-treated and enzymatically-hydrolyzed material. The ethanol concentration was 3.7 mg/mL higher than the concentration obtained for the hydrolysate subjected to enzymatic hydrolysis only. This result confirms the desirability of conducting alkaline pre-treatment.

**Table 8.** Sugar content in hydrolysates and composition of fermentation broth.

Feed for Fermentation	Sugar Composition (mg/mL)	Before Fermentation	After Fermentation	Bioethanol (mg/mL)	Glycerin (mg/mL)	Succinic Acid (mg/mL)	Acetic Acid (mg/mL)	Methanol (mg/mL)
Pure glucose	Glucose	3 g	0	14.83	2.92	0.43	0.48	0
EH	Glucose	10.1	0	5.1	1.14	0.43	0.33	0.39
	Xylose	0.92	0					
	Mannose	0.11	0					
	Arabinose	0.08	0.08					
	Cellobiose	82	0					
	Galactose	1.4	1.32					
AIEH	Glucose	15.06	0	8.8	1.67	0.71	0.49	0.096
	Xylose	0.99	0					
	Mannose	0.03	0					
	Arabinose	0.13	0.13					
	Cellobiose	0.77	0					
	Galactose	1.4	1.12					
AcH	Glucose	13.4	0	6.4	2.35	1.9	1.34	0
	Xylose	3.95	2.78					
	Mannose	0.45	0					
	Arabinose	0.21	0.21					
	Cellobiose	0.53	0.21					
	Galactose	0.8	0.76					
AlAcH	Glucose	8.3	0	4.8	1.21	1.2	0.3	0
	Xylose	0.89	0.32					
	Mannose	0.12	0					
	Arabinose	0	0					
	Cellobiose	0.35	0.03					
	Galactose	0.47	0.39					
AcHD	Glucose	13.3	0	6.7	2.04	1.4	1.18	0.99
	Xylose	3.13	2.75					
	Mannose	0.11	0					
	Arabinose	0.08	0.08					
	Cellobiose	0.53	0.33					
	Galactose	0.7	0.7					
AlAcHD	Glucose	8.4	0	4.9	1.1	1.08	0.33	0.15
	Xylose	0.95	0.65					
	Mannose	0.03	0					
	Arabinose	0.13	0.13					
	Cellobiose	0.42	0.04					
	Galactose	0.44	0.44					



The beneficial effect of alkaline treatment described above does not occur when the saccharification is carried out by means of acid hydrolysis. Higher ethanol yield  $0.21 \text{ g/g}_{\text{biomass}}$  was obtained for the process where the untreated material was hydrolyzed with acid (AcH) compared to the fermentation carried out on the material after two-stage treatment (alkaline pre-treatment and acid hydrolysis, AlAcH), where the bioethanol yield was  $0.16 \text{ g/g}_{\text{biomass}}$ . The given low yield may be due to the large weight loss (48%) as a result of the pre-alkaline treatment. Obtained results of alcoholic fermentation prove that using the alkaline pre-treatment prior to acid hydrolysis is pointless.

In addition, fermentation was carried out on acidic hydrolysates after the removal of fermentation inhibitors. Despite effective detoxification, no significant increase in ethanol yield was obtained. For the fermentation of AcHD hydrolysate,  $0.3 \text{ mg/mL}$  more ethanol was obtained than for AcH, while for AlAcHD, the increase in ethanol was even smaller and was only  $0.1 \text{ mg/mL}$  more than for the AlAcH fermentation.

In other studies, using *Saccharomyces cerevisiae*, partial removal of inhibitors (vanillic acid, furfural, acetic acid, feluric acid, syringaldehyde) allowed to obtain a concentration of ethanol higher by  $0.27 \text{ g/g}_{\text{biomass}}$  [8]. The detoxification by extraction with a mixture of trialkylamine, octanol, and kerosene significantly improved the efficiency of obtained bioethanol ( $21.8 \text{ mg/mL}$ ), in comparison with the ethanol yield obtained from the hydrolysate without detoxification ( $3.79 \text{ mg/mL}$ ) [46]. Despite the poorer results obtained for acid compared to enzymatic hydrolysis, acid hydrolysis may be an attractive method of one-stage hydrolysis prior to alcoholic fermentation. It is mainly related to the low cost of acid compared to the enzyme cost and short preparation time of the fermentation feed material. Hydrolysates from waste lignocellulosic biomass, obtained by using phosphoric acid during hydrolysis, may enrich the process feed for industrial processes of alcoholic fermentations of starch-rich substrates. This approach would increase the share of second-generation biofuels. In addition, when neutralized with phosphoric acid, e.g., with ammonia, a good medium for yeast is obtained, which promotes a reduction in the waste generation from the use of the acid during the saccharification.

Studies on the alcoholic fermentation of acid hydrolysates [1] were also carried out using dilute sulfuric acid. Obtained hydrolysates were rich mainly in hemicellulose hydrolysis products (arabinose and glucose), and the fermentation of these compounds allowed obtaining ethanol ( $0.23 \text{ g/g}_{\text{biomass}}$ ).

The results of fermentation on hydrolysates (Figure 11) indicate that glucose is completely digested within the first 5 h of the process. During the fermentation of AlEH and EH hydrolysates, cellobiose is also digested, however, a significant loss of concentration of this disaccharide is observed only after complete consumption of glucose. Additionally, xylose undergoes digestion only after the complete digestion of glucose by yeast. Total xylose consumption was observed after 24 hours of fermentation. The order of sugar digestion described above is confirmed by other papers indicating that digestion of xylose by *Saccharomyces cerevisiae* is possible only after complete consumption of glucose [3]. Interestingly, the yeast *Saccharomyces cerevisiae* D5A, during the fermentation of hydrolyzed trunks of palm trees, was not able to digest xylose [6].

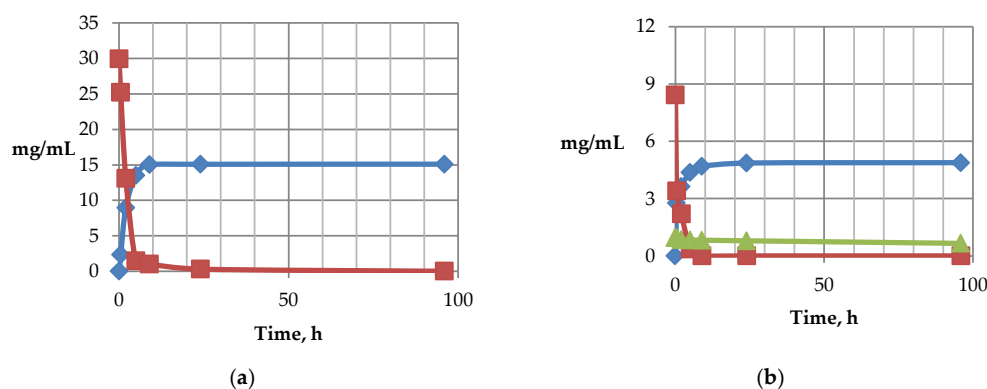
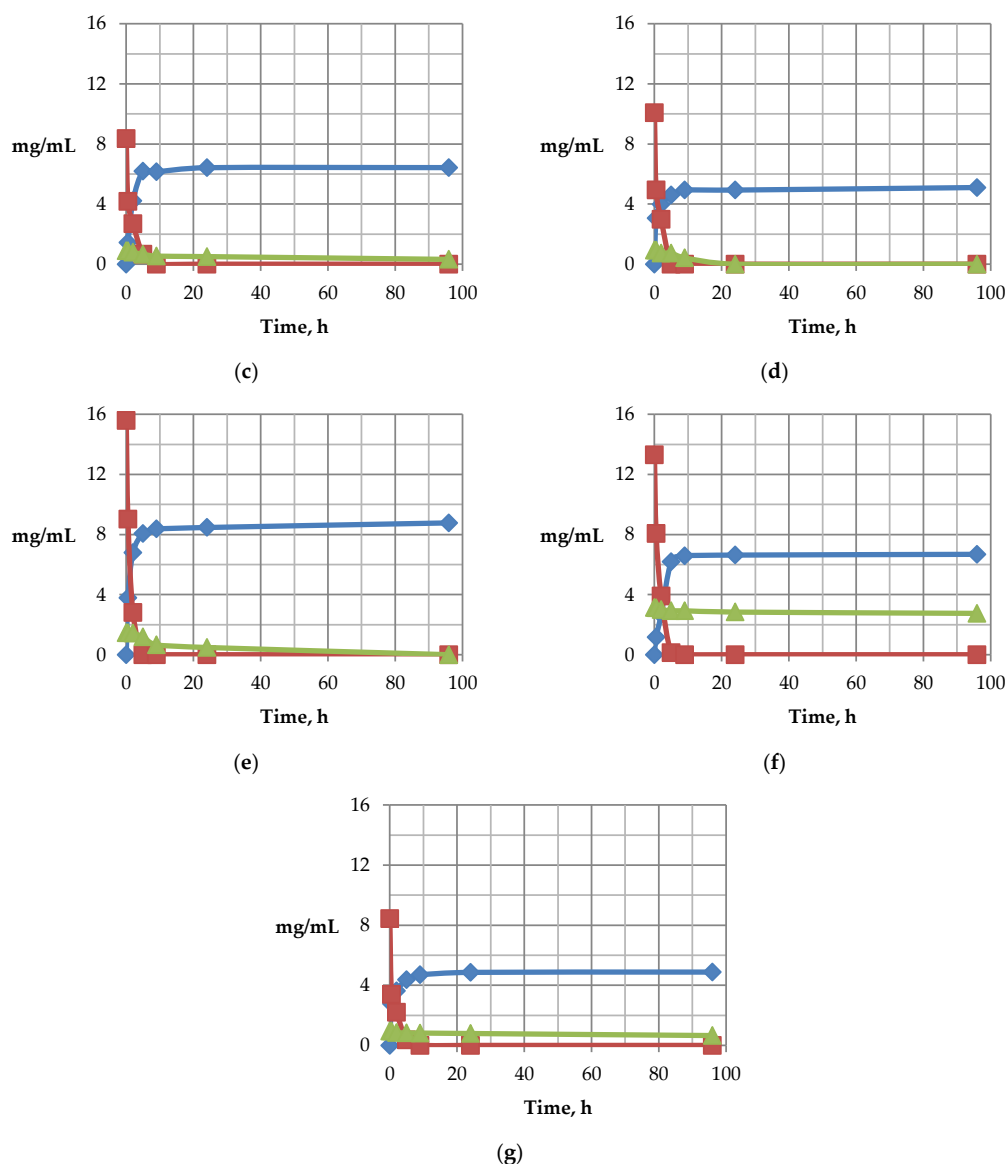


Figure 11. Cont.





**Figure 11.** Courses of bioethanol fermentation presenting bioethanol yield (blue) and consumption of glucose (red) and xylose (green) in function of time from following substrates: (a) pure glucose; (b) AcH; (c) AlAcH; (d) EH; (e) AlEH; (f) AcHD; and (g) AlAcHD.

The research results show that cellobiose and xylose were not completely digested by yeast during fermentation on hydrolysates, both after one-step acid hydrolysis (AcH) and after acid hydrolysis preceded by alkaline pre-treatment (AlAcH). Only 30% and 64% of xylose, respectively, were converted into fermentation products. This result can be explained by the presence of fermentation inhibitors in AcH and AlAcH hydrolysates, however, even after removal of inhibitors (AcHD and AlAcHD), the fermentation of xylose does not proceed completely. It turns out that the presence of inhibitors affects the utilization of xylose in the alcoholic fermentation from *Pichia stipitis* CBS 5776. The results presented in [46] indicate that the xylose from the detoxified hydrolysate was digested completely after 60 h, and during the fermentation on the hydrolysate without detoxification only 15% of xylose was digested. The results of other studies indicate that the digestibility of xylose depends on its initial concentration in the hydrolysate and on the ratio of glucose to xylose. The highest conversion degree of xylose (83.2%) to ethanol and xylitol was achieved when the xylose concentration was about

5.5 times higher than the glucose concentration. A much worse result (20%) was achieved when the fermentation broth contained three times more glucose [1].

Arabinose and galactose, regardless of the method and conditions of biomass pre-treatment and hydrolysis, are not consumed during the fermentation. The inability of *S. cerevisiae* to metabolize arabinose has been previously demonstrated in [1]. In addition, besides bioethanol, other typical byproducts were identified including glycerin, acetic acid, succinic acid, and methanol (Table 8). Table 9 presents a summary of the research results from this work compared to other published results regarding similar biomass types.

**Table 9.** Summary of investigations compared to other published results.

Microorganisms	Type and Method of Pre-Treatment	Hydrolysis Conditions	Biomass Concentration % (w/v)	Ethanol Yield g/L	Ethanol Efficiency	Ref.
<i>Saccharomyces cerevisiae</i>	Hydrothermal 180 °C, 30 min	30 FPU/g, 45 °C	3% wheat straw	14.84	82.4%	[2]
<i>Kluyveromyces</i> sp.	0.5% NaOH 121 °C, 30 min	9 FPU/g substrate	10% wheat straw	18.29	79.64%	[47]
<i>Kluyveromyces marxianus</i> CECT 10875	Steam explosion 100 °C, 2 min and laccase 10 IU/g 50 °C, 24 h	Cellulase 5 FPU/g IB-glucosidase 5 IU/g 50 °C, 72 h	10% TS (w/v) wheat straw	12.7	67.1%	[48]
<i>Saccharomyces cerevisiae</i> (T0936)	0.5% H <sub>2</sub> SO <sub>4</sub> 185 °C, 8 min	12 FPU/g, 50 °C, 24 h	10% SS wheat straw	30.3	83%	[7]
<i>Saccharomyces cerevisiae</i> Ethanol Red <sup>®</sup> as	Steam explosion 200 °C, 7 min	15 FPU, 37 °C, 60 h Spezyme <sup>®</sup> CP/g cellulose	15% (w/v) triticale	29.6	81.3%	[49]
<i>Saccharomyces cerevisiae</i>	Without pre-treatment	85% H <sub>3</sub> PO <sub>4</sub> , 0.5 h, 100 °C	3%	AcH 6.4 AcHD 6.7	AcH 88.5% AcHD 93.9%	This study
<i>Saccharomyces cerevisiae</i>	6% NaOH, 6 h, 65 °C	85% H <sub>3</sub> PO <sub>4</sub> , 0.5 h, 100 °C	3%	AlAcH 4.8 AlAcHD 4.9	AlAcH 44.3% AlAcHD 45.0%	This study
<i>Saccharomyces cerevisiae</i>	Without pre-treatment	Immobilized enzymes 24 h, 42 °C	3%	5.1	70.4%	This study
<i>Saccharomyces cerevisiae</i>	6% NaOH, 6 h, 65 °C	Immobilized enzymes 24 h, 42 °C	3%	8.8	80.5%	This study

Alcoholic fermentation of sugars in the presence of yeast belongs to the rapidly-growing industry and is used in brewing, viticulture, distilling, candling of fruit, yeast production, bread, as well as in environmental protection. This process involves the oxidation of carbohydrates by appropriate yeast strains, resulting in the formation of ethyl alcohol, used for food and industrial purposes. The raw materials used in the fermentation industry are divided into three groups. The first includes sugar products: molasses, fruit juices, sugar cane, the second: starch materials (rye, barley, and wheat), and the third: cellulose products (wood and sulphite waste liquids). Yeast contains enzymes that hydrolyze disaccharides to simple sugars. Only four hexoses are fermented under the influence of yeasts: D-glucose, D-mannose, D-fructose, and D-galactose. The last one is hardly fermented and not under the influence of all yeast species. Alcohol as the final fermentation product is isolated by distillation. The yield calculated on the ratio of fermenting sugars is about 90%.

### 3. Materials and Methods

#### 3.1. Chemical Reagents and Enzymes

- Chemical reagents (sodium hydroxide, phosphoric acid 85%, sulfuric acid 99.5%, hydrochloric acid, calcium oxide, glucose, and (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> are purity grade purchased from Chempur, (Piekary Śląskie, Poland);
- Chemical standards (cellobiose, glucose, mannose, arabinose, xylose, galactose, furfural, HMF, acetic acid, vaniline, succinic acid, lactic acid, and glycerin) are of analysis grade purity purchased from Sigma Aldrich, (Poznań, Poland);

- Demineralized water for HPLC analysis was filtered through a 0.45  $\mu\text{m}$  filter;
- Viscozyme L from the *Aspergillus* sp. Novozyme Corp. (Sigma Aldrich, Poznań, Poland) enzyme complex contains a great variety of carbohydrases: arabinase, cellulase,  $\beta$ -glucanase, hemicellulase, and xylanase  $T_{\text{opt}}$  35–40  $^{\circ}\text{C}$ , pH 5–6.5;
- Glucosidase  $\geq 750$  U/g from *Aspergillus* sp. was acquired from Novozyme Corp. (Sigma Aldrich, Poznań, Poland),  $T_{\text{opt}}$  35–45  $^{\circ}\text{C}$ , pH 4–6.5;
- Cellulase  $\geq 800$  U/g from *Aspergillus niger*, was acquired from Novozyme Corp. (Sigma Aldrich, Poznań, Poland)  $T_{\text{opt}}$  30–40  $^{\circ}\text{C}$ , pH 4–5.8;
- Cellulase  $\geq 700$  U/g from *Trichoderma Reesei* ATCC 26921 was acquired from Novozyme Corp. (Sigma Aldrich, Poznań, Poland);
- *Saccharomyces cerevisiae* (Biomaxima, Gdańsk, Poland);
- *Saccharomyces carlsbergensis* SA23 (ATCC, Manassas, VA, USA);
- Diatomite (Chempur, Piekary Śląskie, Poland);
- Phosphate buffer 0.1 M pH 5.7–8.0 (13.9 g  $\text{NaH}_2\text{PO}_4$  + 35.85 g  $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$ , 500 mL water);
- McIlvaine buffer, pH 4.5 (mix 44.1 mL with 0.02 M  $\text{Na}_2\text{HPO}_4$  with 55.9 mL 0.01 M citric acid and filled to 1000 mL with distilled water);

### 3.2. Biomass Preparation

The air-dried lignocellulosic biomass was milled using a RETSCH Ultra Centrifugal Mill ZM 200 mill (Katowice, Poland). During the milling, a 0.75 mm diameter screen was used. The material after grinding was stored in sealed containers at room temperature. Before the alkaline pre-treatment, the material was dried in a laboratory dryer at 105  $^{\circ}\text{C}$  for 4 h and then stored in a desiccator with the drying substance (NaOH).

### 3.3. Alkaline Pre-Treatment

The alkaline pre-treatment of lignocellulosic biomass was carried out using sodium hydroxide. The reactions were carried out in 250 mL sealed vessels, into which 4 g of milled and minced lignocellulosic biomass and 80 mL of catalyst solution were introduced. The dishes were placed in a shaking water bath. The pre-treatment was carried out at a constant temperature of 65  $^{\circ}\text{C}$  for 6 h for catalyst concentrations (NaOH) of 1%, 6%, and 11%. The reaction mixture was then neutralized with a hydrochloric acid solution, poured into centrifuge tubes, and centrifuged and filtered through a Buchner funnel to separate the precipitate from the solution. The precipitate was then washed three times with water and then twice with acetone. The precipitate was dried for 24 h at 105  $^{\circ}\text{C}$  and placed in a desiccator with a drying substance.

### 3.4. Acid Hydrolysis

The acid hydrolysis processes performed before and after the alkaline pre-treatment were carried out using 85% phosphoric acid. The reactions were carried out in 100 mL sealed vessels, into which 0.5 g of lignocellulosic biomass and 10 mL of catalyst solution were introduced. The vessels were placed in a thermostated shaker. The hydrolysis was carried out at the time and temperature given in Table 4. For optimization purposes, the reaction mixture was diluted to a volume of 80 mL and neutralized with calcium carbonate. For fermentation, the mixture was diluted three times, then partially neutralized with phosphate acid with calcium carbonate. The calcium phosphate formed during the neutralization was filtered off and the hydrolysate was subjected to fermentation.

### 3.5. Enzymatic Hydrolysis

Milled and minced lignocellulosic biomass (0.2 g) after alkaline pre-treatment was added to the flasks and supplemented with a suspension of cellulolytic enzymes immobilized on diatomite to 10 mL. The reaction flasks were incubated in a thermostated shaker at 42  $^{\circ}\text{C}$  for 24 h. Subsequently, samples

were taken, the enzyme-containing bed was separated by centrifugation and filtration, and the contents of monosaccharides and glucose content were analyzed. Control experiments were carried without the addition of lignocellulosic material. To avoid non-linear changes of the process, buffers, such as McIlvaine or phosphate buffer, must be used. Immobilized enzyme preparation requires placing 20 mL of enzymatic solution (Viscozyme L, Glucosidase 0.9:0.1 *m/m* to 1:0) in a 50 mL beaker with 2.5 g dry diatomite and stirring the solution for 1 h at low speed (magnetic stirrer) at room temperature. The diatomite with the immobilized enzyme must be washed in a column with a small amount (about 10 mL) of McIlvaine buffer. After a few minutes the bed stabilizes. Next, the diluted diatomite must be stored under a layer of buffer (about 5 mm). To use the enzyme hydrolysis preparations, the bed should be shaken and then introduced to the solid residue of the lignocellulosic biomass solution after alkaline pre-treatment.

### 3.6. Detoxification of Hydrolysates

A SPE-C18 Polar Plus column (WITKO, Łódź, Poland) with a bed length of 10 cm was used for detoxification, attached to a receiver chamber connected to a vacuum pump. A hydrolysate solution was introduced into the column and passed through the bed under vacuum. The collected eluate was subjected to HPLC analysis in terms of the composition and content of sugars and inhibitors.

### 3.7. Ethanol Fermentation

A solution containing 0.04 g of  $(\text{NH}_4)_2\text{HPO}_4$ , 0.5 g of glucose, and 7 g of mixture of *Saccharomyces cerevisiae* and *Saccharomyces carlbergensis* SA23 in 200 mL of water was prepared. The mixture of given *Saccharomyces* strains is able to consume xylose [50]. The solution was incubated at 37 °C for 2 h. Twenty milliliters of grout was added to fermentation broths prepared as described in Table 4 and filled to 100 mL with water. Fermentation was carried out at 37 °C for one week. Flasks were plugged with stoppers provided with fermentation tubes filled with water.

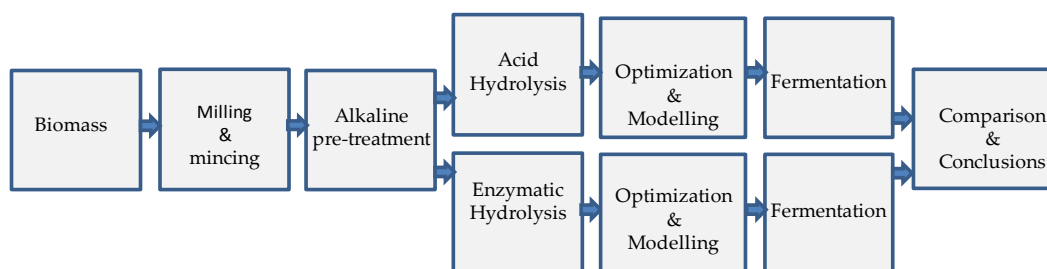
### 3.8. Methods

- Total solids, ash, and extractives of raw triticale straw were determined according to the National Renewable Energy Laboratory (NREL) analytical procedures [51–54].
- The composition and analysis of sugar and lignin content in the native material and after alkaline hydrolysis were determined using the NREL procedure [52]. The content of cellulose and hemicellulose was determined by HPLC with a Rezex Pb<sup>2+</sup> column (300 × 7.8 mm, 8 μm) (Phenomenex, Torrance, CA, USA) and RID detector (Knauer, Berlin, Germany). Water with a flow rate of 0.6 mL/min was used as the eluent.
- The presence and concentration of fermentation inhibitors (furfural, 5-HMF, levulinic acid) were determined by HPLC with a Shodex SH1011 column (300 × 7.8 mm, 8 μm) (Showa Denko, Toyama, Japan) and UV-VIS DAD (Merck-Hitachi, Düsseldorf, Germany) and RID detectors (Knauer, Berlin, Germany). The mobile phase was 5 mM H<sub>2</sub>SO<sub>4</sub> solution. The composition of fermentation broths was determined by HPLC with the Shodex SH1011 column and UV-VIS DAD and RID detectors. The mobile phase was 5 mM H<sub>2</sub>SO<sub>4</sub> solution.
- The concentration of total phenolic compounds was determined by UV-VIS (HP, San Diego, CA, USA). The calibration curve was made for vanillin. The absorbance measurement was made at 280 nm. To obtain the sum of the phenolic compounds, the concentrations of furfural and 5-HMF (determined by HPLC) were subtracted from the concentrations determined by the UV-VIS method.

### 3.9. Design of Experiments and Data Analysis

The experiments described in this paper were realized according to the general scheme presented in Figure 12. Symbols of variables are given in accordance to those used in Tables 4 and 7. Experiments regarding

the conditions of alkaline pre-treatment, as well as acid or enzymatic hydrolysis, were planned and realized according to Box-Behnken design for three factors. Table 10 presents the input variables for a given coding level.



**Figure 12.** Steps of lignocelulosic biomass processing towards bioethanol.

**Table 10.** Input variables for the Box-Behnken design.

Variable	Symbol	Coding Level		
		−1	0	1
NaOH concentration, %	$X_1$	1	6	11
Temperature of acid hydrolysis, °C	$X_2$	60	90	120
Time of acid hydrolysis, h	$X_3$	0.50	4.25	8.00
Viscozyme L content, %	$X_4$	0.90	0.95	1.00
Time of enzymatic hydrolysis, h	$X_5$	8	16	24

Data analysis and related calculations were performed using R Studio Desktop (version 1.0.143). Presentation of the results in the form of response surface as a function of investigated parameters was prepared using Microsoft Excel 2007.

### 3.10. Calculations

Biomass recovery was calculated from Equation (3):

$$BR (\%) = (\text{Mass of pre-treated biomass (g)}/\text{Mass of feed biomass (g)}) \times 100\% \quad (3)$$

Glucan and xylan recovery was calculated as follows:

$$\text{Glucan/xylan recovery} = BR (\%) \times (C_{\text{glucan/xylan pretreated biomass}}/C_{\text{glucan/xylan original biomass}}) \quad (4)$$

The delignification degree (i.e., lignin removal) was calculated from Equation (5):

$$LR (\%) = 100\% - (BR (\%) \times (C_{\text{lignin pretreated}}/C_{\text{lignin original biomass}})) \quad (5)$$

where C is lignin content (wt %).

The theoretical ethanol yield was calculated according to the NREL standard procedure [55] from the following formula:

$$\text{Theoretical EtOH yield \%} = (\text{EtOH produced}/(0.51 \times f \times \text{Biomass} \times 1.111)) \times 100\% \quad (6)$$

where EtOH produced is the ethanol concentration at the end of the fermentation (g/L). The term  $0.51 \times f \times \text{Biomass} \times 1.111$  corresponds to the theoretical ethanol concentration, where Biomass is the dry biomass weight concentration at the beginning of the fermentation (g/L);  $f$  is the cellulose fraction of dry biomass (g/g); 0.51 is the conversion factor for glucose to ethanol based on the stoichiometric biochemistry of yeast, and 1.111 is the conversion factor for cellulose to equivalent glucose.

**Acknowledgments:** This work was carried out within the framework of the project “Studies of alkaline hydrolysis of lignocellulosic biomass and conversion conditions of hydrolyzed products to biogas”, supported financially by the National Science Center through grant UMO-2014/13/B/ST8/04258. We have received funds for covering the costs to publish in open access.

**Author Contributions:** Rafał Łukajtis and Karolina Kucharska conceived and designed the experiments; Rafał Łukajtis, Karolina Kucharska, Edyta Słupek, Katarzyna Wychodnik, and Paulina Nowak performed the experiments; Karolina Kucharska and Katarzyna Wychodnik performed the ethanol fermentation, Piotr Rybarczyk analyzed the data; Marian Kamiński provided reagents/materials/analysis tools; and Iwona Hołowacz, Rafał Łukajtis, Karolina Kucharska, and Piotr Rybarczyk wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## References

1. Cortivo, P.R.D.; Hickert, L.R.; Hector, R.; Ayub, M.A.Z. Fermentation of oat and soybean hull hydrolysates into ethanol and xylitol by recombinant industrial strains of *Saccharomyces cerevisiae* under diverse oxygen environments. *Ind. Crops Prod.* **2018**, *113*, 10–18. [[CrossRef](#)]
2. Ruiz, H.A.; Silva, D.P.; Ruzene, D.S.; Lima, L.F.; Vicente, A.A.; Teixeira, J.A. Bioethanol production from hydrothermal pretreated wheat straw by a flocculating *Saccharomyces cerevisiae* strain-Effect of process conditions. *Fuel* **2012**, *95*, 528–536. [[CrossRef](#)]
3. Zhu, J.Q.; Li, X.; Qin, L.; Li, W.C.; Li, H.Z.; Li, B.Z.; Yuan, Y.J. In situ detoxification of dry dilute acid pretreated corn stover by co-culture of xylose-utilizing and inhibitor-tolerant *Saccharomyces cerevisiae* increases ethanol production. *Bioresour. Technol.* **2016**, *218*, 380–387. [[CrossRef](#)] [[PubMed](#)]
4. Białas, W.; Szymanowska, D.; Grajek, W. Fuel ethanol production from granular corn starch using *Saccharomyces cerevisiae* in a long term repeated SSF process with full stillage recycling. *Bioresour. Technol.* **2010**, *101*, 3126–3131. [[CrossRef](#)] [[PubMed](#)]
5. Nilsson, A. *Control of Fermentation of Lignocellulosic Hydrolysates*; Lund University: Lund, Sweden, 2001.
6. Jung, Y.H.; Kim, I.J.; Kim, J.J.; Oh, K.K.; Han, J.I.; Choi, I.G.; Kim, K.H. Ethanol production from oil palm trunks treated with aqueous ammonia and cellulase. *Bioresour. Technol.* **2011**, *102*, 7307–7312. [[CrossRef](#)] [[PubMed](#)]
7. Ishola, M.M.; Ylittero, P.; Taherzadeh, M.J. Co-Utilization of glucose and xylose for enhanced lignocellulosic ethanol production with reverse membrane bioreactors. *Membranes* **2015**, *5*, 844–856. [[CrossRef](#)] [[PubMed](#)]
8. Tavva, S.S.M.D.; Deshpande, A.; Durbha, S.R.; Palakollu, V.A.R.; Goparaju, A.U.; Yechuri, V.R.; Bandaru, V.R.; Muktinutalapati, V.S.R. Bioethanol production through separate hydrolysis and fermentation of *Parthenium hysterophorus* biomass. *Renew. Energy* **2016**, *86*, 1317–1323. [[CrossRef](#)]
9. Alvira, P.; Tomas-Pejo, E.; Ballesteros, M.; Negro, M.J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour. Technol.* **2010**, *101*, 4851–4861. [[CrossRef](#)] [[PubMed](#)]
10. Brodeur, G.; Yau, E.; Badal, K.; Collier, J.; Ramachandran, K.B.; Ramakrishnan, S. Chemical and Physicochemical Pretreatment of Lignocellulosic Biomass: A Review. *Enzyme Res.* **2011**, *2011*, e787532. [[CrossRef](#)] [[PubMed](#)]
11. Kumar, G.; Bakonyi, P.; Periyasamy, S.; Kim, S.H.; Nemestóthy, N.; Bélafi-Bakó, K. Lignocellulose biohydrogen: Practical challenges and recent progress. *Renew. Sustain. Energy Rev.* **2015**, *44*, 728–737. [[CrossRef](#)]
12. Kumar, A.K.; Sharma, S. Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review. *Bioresour. Bioprocess.* **2017**, *4*, 7–14. [[CrossRef](#)] [[PubMed](#)]
13. Bali, G.; Meng, X.; Deneff, J.L.; Sun, Q.; Ragauskas, A.J. The Effect of Alkaline Pretreatment Methods on Cellulose Structure and Accessibility. *ChemSusChem* **2015**, *8*, 275–279. [[CrossRef](#)] [[PubMed](#)]
14. Abudi, Z.N.; Hu, Z.; Xiao, B.; Abood, A.R.; Rajaa, N.; Laghari, M. Effects of pretreatments on thickened waste activated sludge and rice straw co-digestion: Experimental and modeling study. *J. Environ. Manag.* **2016**, *177*, 213–222. [[CrossRef](#)] [[PubMed](#)]
15. Khare, S.K.; Pandey, A.; Larroche, C. Current perspectives in enzymatic saccharification of lignocellulosic biomass. *Biochem. Eng. J.* **2015**, *102*, 38–44. [[CrossRef](#)]

16. Sun, Y.; Cheng, J. Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresour. Technol.* **2002**, *83*, 1–11. [[CrossRef](#)]
17. Sartori, T.; Tibolla, H.; Prigol, E.; Colla, L.M.; Costa, J.A.V.; Bertolin, T.E. Enzymatic saccharification of lignocellulosic residues by cellulases obtained from solid state fermentation using *Trichoderma viride*. *BioMed Res. Int.* **2015**, *2015*, 342716. [[CrossRef](#)] [[PubMed](#)]
18. Rabemanantsoa, H.; Saka, S. Various pretreatments of lignocellulosics. *Bioresour. Technol.* **2016**, *199*, 83–91. [[CrossRef](#)] [[PubMed](#)]
19. Selig, M.J.; Tucker, M.P.; Law, C.; Doeppke, C.; Himmel, M.E.; Decker, S.R. High throughput determination of glucan and xylan fractions in lignocelluloses. *Biotechnol. Lett.* **2011**, *33*, 961–967. [[CrossRef](#)] [[PubMed](#)]
20. Trzcinski, A.P.; Stuckey, D.C. Contribution of acetic acid to the hydrolysis of lignocellulosic biomass under abiotic conditions. *Bioresour. Technol.* **2015**, *185*, 441–444. [[CrossRef](#)] [[PubMed](#)]
21. Yücel, H.G.; Aksu, Z. Ethanol fermentation characteristics of *Pichia stipitis* yeast from sugar beet pulp hydrolysate: Use of new detoxification methods. *Fuel* **2015**, *158*, 793–799. [[CrossRef](#)]
22. Agbogbo, F.K.; Wenger, K.S. Production of ethanol from corn stover hemicellulose hydrolyzate using *Pichia stipitis*. *J. Ind. Microbiol. Biotechnol.* **2007**, *34*, 723–727. [[CrossRef](#)] [[PubMed](#)]
23. Kupiainen, L.; Ahola, J.; Tanskanen, J. Kinetics of Formic Acid-catalyzed Cellulose Hydrolysis. *BioResources* **2014**, *9*, 2645–2658. [[CrossRef](#)]
24. Sivagurunathan, P.; Kumar, G.; Mudhoo, A.; Rene, E.R.; Saratale, G.D.; Kobayashi, T.; Xu, K.; Kim, S.H.; Kim, D.H. Fermentative hydrogen production using lignocellulose biomass: An overview of pre-treatment methods, inhibitor effects and detoxification experiences. *Renew. Sustain. Energy Rev.* **2017**, *77*, 28–42. [[CrossRef](#)]
25. Agbor, V.B.; Cicek, N.; Sparling, R.; Berlin, A.; Levin, D.B. Biomass pretreatment: Fundamentals toward application. *Biotechnol. Adv.* **2011**, *29*, 675–685. [[CrossRef](#)] [[PubMed](#)]
26. Da Silva, C.; De Menezes Silva Conde, M.; Longhi-Wagner, H.M. Olyreae (Poaceae: Bambusoideae) of Marambaia, Rio de Janeiro, Brazil; Olyreae (Poaceae Bambusoideae) da Marambaia, Rio Janeiro, Bras. *Rodriguésia* **2012**, *63*, 357–372. [[CrossRef](#)]
27. Lau, M.W.; Gunawan, C.; Balan, V.; Dale, B.E. Comparing the fermentation performance of *Escherichia coli* KO11, *Saccharomyces cerevisiae* 424A (LNH-ST) and *Zymomonas mobilis* AX101 for cellulosic ethanol production. *Biotechnol. Biofuels* **2010**, *3*, 11. [[CrossRef](#)] [[PubMed](#)]
28. Garcia Sanchez, R.; Karhumaa, K.; Fonseca, C.; Sánchez Nogué, V.; Almeida, J.R.; Larsson, C.U.; Bengtsson, O.; Bettiga, M.; Hahn-Hägerdal, B.; Gorwa-Grauslund, M.F. Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotechnol. Biofuels* **2010**, *3*, 13. [[CrossRef](#)] [[PubMed](#)]
29. Wikandari, R.; Millati, R.; Taherzadeh, M.J. Pretreatment of Lignocelluloses with Solvent *N*-Methylmorpholine *N*-oxide. In *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery*; Elsevier Inc.: São Paulo, Brazil, 2016; ISBN 9780128025611.
30. García, M.; Gonzalo, A.; Sánchez, L.; Arauzo, J.; Simoes, C. Methanolysis and ethanolysis of animal fats: A comparative study of the influence of alcohols. *Chem. Ind. Chem. Eng. Q.* **2011**, *17*, 91–97. [[CrossRef](#)]
31. Silva, J.P.A.; Mussatto, S.I.; Roberto, I.C. The influence of initial xylose concentration, agitation, and aeration on ethanol production by *Pichia stipitis* from rice straw hemicellulosic hydrolysate. *Appl. Biochem. Biotechnol.* **2010**, *162*, 1306–1315. [[CrossRef](#)] [[PubMed](#)]
32. Alrumman, S.A. Enzymatic saccharification and fermentation of cellulosic date palm wastes to glucose and lactic acid. *Braz. J. Microbiol.* **2016**, *47*, 110–119. [[CrossRef](#)] [[PubMed](#)]
33. Jiang, Y.; Li, X.; Wang, X.; Meng, L.; Wang, H.; Peng, G.; Wang, X.; Mu, X. Effective saccharification of lignocellulosic biomass over hydrolysis residue derived solid acid under microwave irradiation. *Green Chem.* **2012**, *14*, 2162–2167. [[CrossRef](#)]
34. Hasegawa, I.; Khoo, T.H.; Mae, K. Direct saccharification of lignocellulosic biomass by hydrolysis with formic acid solution. *Green Process. Synth.* **2013**, *2*, 143–149. [[CrossRef](#)]
35. Scordia, D.; Cosentino, S.L.; Jeffries, T.W. Enzymatic hydrolysis, simultaneous saccharification and ethanol fermentation of oxalic acid pretreated giant reed (*Arundo donax* L.). *Ind. Crops Prod.* **2013**, *49*, 392–399. [[CrossRef](#)]
36. E Silva, C.F.L.; Schirmer, M.A.; Maeda, R.N.; Barcelos, C.A.; Pereira, N. Potential of giant reed (*Arundo donax* L.) for second generation ethanol production. *Electron. J. Biotechnol.* **2015**, *18*, 10–15. [[CrossRef](#)]

37. Cotana, F.; Barbanera, M.; Foschini, D.; Lascaro, E.; Buratti, C. Preliminary optimization of alkaline pretreatment for ethanol production from vineyard pruning. *Energy Procedia* **2015**, *82*, 389–394. [CrossRef]
38. Kuhad, R.C.; Gupta, R.; Khasa, Y.P.; Singh, A. Bioethanol production from *Lantana camara* (red sage): Pretreatment, saccharification and fermentation. *Bioresour. Technol.* **2010**, *101*, 8348–8354. [CrossRef] [PubMed]
39. Chaudhary, G.; Singh, L.K.; Ghosh, S. Alkaline pretreatment methods followed by acid hydrolysis of *Saccharum spontaneum* for bioethanol production. *Bioresour. Technol.* **2012**, *124*, 111–118. [CrossRef] [PubMed]
40. Taherzadeh, M.J.; Karimi, K. Fermentation Inhibitors in Ethanol Processes and Different Strategies to Reduce Their Effects. In *Biofuels*; Elsevier: São Paulo, Brazil, 2011; pp. 287–311, ISBN 9780123850997.
41. Kim, S.K.; Park, D.H.; Song, S.H.; Wee, Y.J.; Jeong, G.T. Effect of fermentation inhibitors in the presence and absence of activated charcoal on the growth of *Saccharomyces cerevisiae*. *Bioprocess Biosyst. Eng.* **2013**, *36*, 659–666. [CrossRef] [PubMed]
42. Kundu, C.; Trinh, L.T.P.; Lee, H.J.; Lee, J.W. Bioethanol production from oxalic acid-pretreated biomass and hemicellulose-rich hydrolysates via a combined detoxification process. *Fuel* **2015**, *161*, 129–136. [CrossRef]
43. Keshav, P.K.; Shaik, N.; Koti, S.; Linga, V.R. Bioconversion of alkali delignified cotton stalk using two-stage dilute acid hydrolysis and fermentation of detoxified hydrolysate into ethanol. *Ind. Crops Prod.* **2016**, *91*, 323–331. [CrossRef]
44. Lee, H.J.; Lim, W.S.; Lee, J.W. Improvement of ethanol fermentation from lignocellulosic hydrolysates by the removal of inhibitors. *J. Ind. Eng. Chem.* **2013**, *19*, 2010–2015. [CrossRef]
45. Sindhu, R.; Kuttiraja, M.; Prabisha, T.P.; Binod, P.; Sukumaran, R.K.; Pandey, A. Bioresource Technology Development of a combined pretreatment and hydrolysis strategy of rice straw for the production of bioethanol and biopolymer. *Bioresour. Technol.* **2016**, *215*, 110–116. [CrossRef] [PubMed]
46. Zhu, J.; Yong, Q.; Xu, Y.; Yu, S. Detoxification of corn stover prehydrolyzate by trialkylamine extraction to improve the ethanol production with *Pichia stipitis* CBS 5776. *Bioresour. Technol.* **2011**, *102*, 1663–1668. [CrossRef] [PubMed]
47. Narra, M.; James, J.P.; Balasubramanian, V. Simultaneous saccharification and fermentation of delignified lignocellulosic biomass at high solid loadings by a newly isolated thermotolerant *Kluyveromyces* sp. for ethanol production. *Bioresour. Technol.* **2015**, *179*, 331–338. [CrossRef] [PubMed]
48. Moreno, A.; Ibarra, D.; Mialon, A.; Ballesteros, M. A Bacterial Laccase for Enhancing Saccharification and Ethanol Fermentation of Steam-Pretreated Biomass. *Fermentation* **2016**, *2*, 11. [CrossRef]
49. Kossatz, H.L.; Rose, S.H.; Viljoen-Bloom, M.; van Zyl, W.H. Production of ethanol from steam exploded triticale straw in a simultaneous saccharification and fermentation process. *Process Biochem.* **2017**, *53*, 10–16. [CrossRef]
50. McMillan, J.D. Xylose Fermentation to Ethanol: A Review. Available online: <https://www.nrel.gov/docs/legosti/old/4944.pdf> (accessed on 4 Mar 2018).
51. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Ash in Biomass: Laboratory Analytical Procedure (LAP)*; Technical Report NREL/TP-510-42622; NREL: Golden, CO, USA, 2008.
52. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples*; Technical Report NREL/TP-510-42623; NREL: Golden, CO, USA, 2008; pp. 1–14.
53. Sluiter, A.; Hames, B.; Hyman, D.; Payne, C.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples*; Technical Report NREL/TP-510-42621; NREL: Golden, CO, USA, 2008; p. 9.
54. Hames, B.; Scarlata, C.; Nrel, A.S. *Determination of Protein Content in Biomass*; Technical Report NREL/TP-510-42625; NREL: Golden, CO, USA, 2008.
55. Resch, M.G.; Baker, J.O.; Nrel, S.R.D. *Low Solids Enzymatic Saccharification of Lignocellulosic Biomass Low Solids Enzymatic Saccharification of Lignocellulosic Biomass Laboratory Analytical Procedure (LAP)*; Technical Report NREL/TP-5100-63351; NREL: Golden, CO, USA, 2015.

