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Optimization of Saccharification Conditions of Lignocellulosic Biomass under Alkaline Pre-Treatment and Enzymatic Hydrolysis

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Abstract: Pre-treatment is a significant step in the production of second-generation biofuels from waste lignocellulosic materials. Obtaining biofuels as a result of fermentation processes requires appropriate pre-treatment conditions ensuring the highest possible degree of saccharification of the feed material. An influence of the following process parameters were investigated for alkaline pre-treatment of *Salix viminalis* L.: catalyst concentration (NaOH), temperature, pre-treatment time and granulation. For this purpose, experiments were carried out in accordance to the Box-Behnken design for four factors. In the saccharification process of the pre-treated biomass, cellulolytic enzymes immobilized on diatomaceous earth were used. Based on the obtained results, a mathematical model for the optimal conditions of alkaline pre-treatment prediction is proposed. The optimal conditions of alkaline pre-treatment are established as follows: granulation 0.75 mm, catalyst concentration 7%, pre-treatment time 6 h and temperature 65 °C if the saccharification efficiency and cost analysis are considered. An influence of the optimized pre-treatment on both the chemical composition and structural changes for six various lignocellulosic materials (energetic willow, energetic poplar, beech, triticale, meadow grass, corncobs) was investigated. SEM images of raw and pre-treated biomass samples are included in order to follow the changes in the biomass structure during hydrolysis.

Keywords: lignocellulosic biomass; monosugars; alkaline pre-treatment; enzymatic hydrolysis; optimization; Box-Behnken design

1. Introduction

Lignocellulosic biomass is a low-cost and renewable material being a substrate to obtain various types of substances. Conversion of lignocellulosic biomass through fermentation processes have a great potential for production of many useful products, e.g. biofuels, biomaterials and biochemicals [1]. The development of the world economy implies the need to use renewable non-edible raw materials for production of second-generation biofules [2]. Both lignocellulosic waste as well as special crops with low requirements for cultivation are well suited to the market. Recently, the food versus fuel debate is focused on elaboration of the balance in agriculture crops and avoiding conflicts between food and biofuel production [3].

Production of second-generation biofuels from lignocellulosic raw materials by fermentative methods requires their processing into fermentable simple sugars. Three main steps influencing the efficiency of the biomass conversion are pre-treatment, enzymatic or acid hydrolysis and fermentation.

The type of the raw material subjected to saccharification must be taken into account during the design of the production process to obtain a well-satisfactory biomass to biofuel conversion degree.

The degree of fermentation depends primarily on the biomass composition and its chemical structure. Lignocellulosic biomass is composed mainly of cellulose, hemicellulose and lignin. Cellulose and hemicellulose are biopolymers that can be hydrolyzed into monosugars and exploited in fermentation processes. Crystalline cellulose is a polymer composed of glucane subunits, which are the source of glucose and amorphous hemicellulose which decomposes to a mixture of pentoses and hexoses, such as arabinose, galactose, glucose, mannose and xylose. Differences in the structure of cellulose and hemicellulose influence the rate and yield of hydrolysis. Acidic depolymerization of crystalline cellulose requires more restrictive conditions than those used during hydrolysis of amorphous hemicellulose. However, enzymatic hydrolysis of cellulose catalyzed by cellulases and of hemicellulose carried out with cocktail of enzymes can be carried out in relatively mild conditions [4–6]. Lignin bonds the cellulose and hemicellulose fibers through a variety of linkages [7]. Moreover, lignin is highly resistant to chemical and enzymatic degradation [8,9]. Therefore, the major problem related to the application of lignocellulose as a substrate in fermentation processes is a destruction of the natural structure of carbohydrate-lignin complex, ensuring the lignin removal prior to hydrolysis [10,11]. A pre-treatment of raw lignocellulosic biomass allows to enhance the accessibility of cellulose to the enzymes by the cell wall disruption, lignin removal, reduction of the cellulose crystallinity, swelling pores in the biomass structure and an increase of the surface area [12,13]. The pre-treatment of lignocellulosic biomass includes physical, chemical, physicochemical and biological methods [14–19]. Combinations of several methods are also proposed, e.g. ultrasound and acid or alkaline pre-treatment [20,21]

The alkaline pre-treatment is a useful method to remove lignin from lignocellulosic materials and to increase the accessibility of biomass structure to reagents.This is related to an increase of the material's surface area. The main advantages of the alkaline hydrolysis are (i)high efficiency in lignin removal; (ii) effective removal of acetyl groups and uronic substitutions from hemicellulose; (iii) the low cost [22]. After the delignification of raw biomass, the obtained pre-treated material can be further processed in the further hydrolysis step. There are two main methods allowing to convert carbohydrate polymers into fermentative monomeric sugars, i.e. acid hydrolysis and enzymatic hydrolysis [22]. The advantages of the latter method are as follows: (i) higher yields and minimal byproducts generation; (ii) mild conditions; (iii) no corrosion issues for equipment [23,24]. The overall biomass conversion effectiveness depends on the efficiency of both abovementioned steps. Therefore, the optimization of both pretreatment and enzymatic hydrolysis are important for the selection of the ranges of boundary conditions and the values of the key process parameters resulting in the highest yields of monosugars.

The efficiency of alkaline pre-treatment depends on the type of reagent used. Various chemicals such as sodium, potassium, calcium or ammonium hydroxide, as well as hydrazine or triethylamine are applied in this process [25–27]. The investigated parameters influencing the delignification include the type of alkaline reagent, its concentration, lignocellulosic biomass loading, time of reaction and temperature [26–29]. Some examples of recent studies on the alkaline pre-treatment of lignocellulosic biomass are given in Table 1.

The degree of cellulose and hemicellulose degradation after enzymatic hydrolysis depends on the applied pre-treatment of lignocellulosic biomass, its structural features, the type of used enzymes, substrate loading, reaction time, pH and temperature [22,28–35]. Examples of selected recent research on enzymatic hydrolysis of alkaline pre-treated biomass are given in Table 2.

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Table 1. Selected research on alkaline pre-treatment of lignocellulosic biomass.

Feedstock	Investigated Parameters	Response	Reference
Corn cobs	C_{NaOH} = 2% wt; t = 80 °C, τ = 6 h; S:L = 1:10 $C_{Ca(OH)2}$ = 1% wt; t = 120 °C, τ = 8 h; S:L = 1:20 $C_{NH3 \cdot H2O}$ = 15% wt; t = 60 °C, τ = 16 h; S:L = 1:20 *w*/*v*	Accessible interior surface area, exterior surface area, pore volume distribution, concentration of glucan, xylan and lignin	[22]
Post-grain harvest sorghum straw	C_{NaOH} = 0–2% *w*/*v*; t = 60; 121 °C, τ = 30–90 min; S:L = 1:10 *w*/*v*	Sugar solubilization, lignin reduction solid losses	[35]
Potato peel residues	C_{NaOH} = 1% *w*/*v*; t = 121 °C, τ = 30 min; S:L = 1:10 *w*/*v*	Cellulose, starch and lignin concentration	[30]
Sweet sorghum bagasse	C_{NaOH} = 0.3–3.7% *w*/*v*; $C_{Ca(OH)2}$ = 0.3–3.7% *w*/*v*; t = 100 °C, τ = 0.3–3.7 h; S:L = 3.3:11.7 *w*/*v*	Biomass conversion, lignin removal, morphological changes	[27]
Coffee pulp	C_{NaOH} = 0–8% *w*/*v*; $C_{Ca(OH)2}$ = 0–8% *w*/*v*; t = 121 °C, τ = 16.5–33.4 min; S:L = 1:5 *w*/*v*	Concentration of lignin, cellulose and hemicellulose	[29]
Poplar wood	$C_{Ca(OH)2}$ = 5% *w*/*v*, t = 25–65 °C, τ = 0–12 weeks	Concentration of lignin, glucan and xylan	[36]
Coastal Bermuda grass	$C_{Ca(OH)2}$ = 2–20% wt; t = 21–121 °C, τ = 8 h; S:L = 1:10	Lignin reduction, total reducing sugars, glucose yield, xylose yield	[26]
Switchgrass	C_{NaOH} = 0.5–2% wt; t = 21–121 °C, τ = 0.25–96 h; S:L = 1:10	Solid recovery, lignin reduction	[37]

S—solid, L—liquid, τ—pre-treatment time, t—temperature.

Table 2. Selected research on enzymatic hydrolysis of alkaline pre-treated lignocellulosic biomass.

Feedstock	Investigated Parameters	Response	Reference
Corn cobs	Accessible interior surface area 7–60 m^2/g, exterior surface area 0.75–2 m^2/g, lignin content 2–18% (wt), τ = 2–24 h	Digestibility at 2 h (%)	[22]
Post-grain harvest sorghum straw	Enzyme combination and its specific activity, enzyme dosage	Glucose concentration (mg/g), xylose concentration (mg/g), the rate of saccharification	[35]
Potato peel residues	t = 30–60 °C, pH = 5–8, substrate concentration 2–10% (*w*/*v*), and surfactant concentration 0–1% (*v*/*v*)	Saccharification yield	[30]
Sweet sorghum bagasse	t = 50 °C, pH = 4.8, τ = 48 h	Total reducing sugars, morphological changes	[27]
Coffee pulp	t = 30 °C, τ = 48 h	Reducing sugars, total reducing sugars, glucose concentration	[29]
Coastal bermudagrass	t = 55 °C, pH = 4.8, τ = 72 h	Total reducing sugars, glucose yield, xylose yield	[26]
Switchgrass	t = 55 °C, pH = 4.8, τ = 72 h	Total reducing sugars, concentration of glucose and xylose	[37]

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The appropriate design of combined alkaline pre-treatment and enzymatic hydrolysis allows to obtain high yields of monosugars in hydrolyzates, even close to theoretical ones. Additionally, low concentrations of substances acting as inhibitors are formed, which is beneficial during further fermentation process. Therefore, the aim of this work is to develop the optimal conditions for producing hydrolyzates from lignocellulosic biomass. The comparison of the concentration of alkaline reagent used in the pre-treatment stage and the selection of the most favorable process parameters to obtain the highest monosugars yield were elaborated using the Box-Behnken experimental design which method is often used for optimization purposes regarding biomass hydrolysis and processing [38]. The values of the investigated alkaline reagent concentration and pre-treatment time were selected on the basis of the range of variables described previously for a wide range of lignocellulosic biomass [39–42]. The influence of the size of lignocellulosic biomass particles on delignification process was also investigated. The influence of the pre-treated materials of various types under different conditions on the concentration of sugars was also studied. The novelty of the paper is related to: -(i) comparison of various biomass feedstock materials in the view of saccharification yield; -(ii) specific investigations regarding the quantitative effect of the biomass fragmentation on the saccharification yield; -(iii) optimization of saccharification conditions with respect to the cost analysis of the pre-treatment operations. Additionally, the proposed model describing the saccharification efficiency is general and may be applied for predicting the sugar feed for various fermentative processes, including i.e. the process design in the perspective of formation of value-added products in biorefineries.

2. Results and Discussion

2.1. Biomass Characterisation

Minced and milled lignocellulosic materials obtained from a variety of species with diversified contents of lignin, cellulose and hemicellulose were investigated. Chosen biomass materials may be classified as wood raw materials (energetic willow *Salix viminalis* L., energetic poplar *Populisindustria*, beech *Fagus* L.) and agricultural waste (wheat straw *Triticum paleas*, corncobs *Zeamays*, meadow grass *Poapratensis*). The composition of the above materials is presented in Table 3. The elemental composition of investigated materials determined with the EDX technique is given in Table A1 in Appendix A.

Table 3. Composition of investigated materials.

Lignocellulosic Material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractives (%)		Ash (%)	Moisture (%)
				Ethanol	Water		
Energetic willow	46.5	15.6	29.4	4.2	6.3	0.6	7.0
Beech	38.6	19.9	26.3	8.5	12.2	0.1	3.3
Energetic poplar	39.5	22.2	26.3	8.4	6.4	0.03	3.5
Triticale	39.1	25.4	22.4	8.6	8.3	1.2	6.2
Meadow grass	27.1	22.6	16.8	14.3	22.4	10.1	2.5
Corn cobs	41.0	22.6	14.1	35.8	32.2	1.6	4.8

2.2. Box-Behnken Experimental Design

An increase of the availability of biomass material for cellulolytic enzymes is the aim of the research on the optimization of the alkaline pre-treatment. Energetic willow was used as the raw material during the optimization step, due to low soil requirements, rapid growth and both popularity and representativeness in the group of the energetic plants. Experiments were carried out according to the Box-Behnken design for four variables (Tables 4 and 5). Based on the preliminary investigations and previous literature data, the following boundary conditions were adopted for the investigated process parameters: catalyst concentration 1% and 7%, temperature 30 and 80 °C, treatment time 0.5 and 7 h, granulation 0.25 and 4 mm. The above-mentioned parameters were assigned as independent variables from X_1 to X_4, according to the values presented in Table 4.

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Table 4. Input variables for the four-factorial Box-Behnken design.

Variable	Symbol	Coding Level		
		−1	**0**	**1**
Catalyst concentration, %	X_1	1	4	7
Temperature, °C	X_2	30	55	80
Pre-treatment time, h	X_3	0.50	3.75	7.00
Granulation, mm	X_4	0.25	2.00	4.00

Table 5. Box-Behnken experimental design for two-step hydrolysis of *Salix Viminalis* L.

No.	C_{NaOH} (% m/v)	Granulation (mm)	t (°C)	τ (h)	G_{eff} (-)	Glucose ($mg/g_{biomass}$)	Sum of Sugars ($mg/g_{biomass}$)
1	4	2	80	7.00	0.343	152.5	194.3
2	4	0.25	80	3.75	0.833	510.6	635.1
3	7	0.25	55	3.75	0.522	362.1	471.9
4	1	2	30	3.75	0.126	65.8	107.9
5	4	2	30	7.00	0.107	57.6	106.0
6	4	4	80	3.75	0.189	108.2	153.9
7	1	4	55	3.75	0.360	18.0	103.1
8	7	4	55	3.75	0.439	256.4	303.0
9	7	2	55	7.00	0.790	425.8	499.9
10	4	2	55	3.75	0.433	235.0	305.2
11	4	4	30	3.75	0.350	16.8	101.1
12	1	2	55	0.50	0.600	28.5	79.5
13	4	0.25	55	0.50	0.517	249.6	301.5
14	4	2	55	3.75	0.558	293.0	366.9
15	4	2	55	3.75	0.491	249.3	345.0
16	4	2	55	3.75	0.457	234.3	314.0
17	4	4	55	0.50	0.133	63.6	100.8
18	4	2	80	0.50	0.275	125.1	162.4
19	4	0.25	55	7.00	0.833	379.3	448.6
20	1	2	80	3.75	0.302	134.1	170.9
21	7	2	80	3.75	0.352	167.4	186.2
22	7	2	30	3.75	0.133	71.2	109.9
23	4	0.25	30	3.75	0.770	46.8	79.4
24	4	4	55	7.00	0.442	185.8	225.5
25	1	2	55	7.00	0.890	44.8	89.3
26	4	2	55	3.75	0.455	245.0	310.9
27	7	2	55	0.50	0.429	173.7	213.2
28	1	0.25	55	3.75	0.600	24.6	53.9
29	4	2	30	0.50	0.320	17.9	32.5

Glucose efficiency (G_{eff}), calculated from Equation (2), was assumed as the model output parameter. The granulation values of 0.25, 2 and 4 mm were selected due to the availability of grinding sieve sizes, deliberately and consciously deciding to deviate from the assumptions of the Box-Behnken experimental design. The values of glucose concentration and the sum of reducing sugars and disaccharides are presented in Table 5. As a result of the saccharification carried out by means of enzymatic hydrolysis, the following sugars, except glucose, were identified in the enzymatic hydrolyzate: cellobiose, xylose, galactose, mannose and arabinose.

The composition of the biomass, the degree of fragmentation and the pre-treatment parameters (i.e., temperature, type and concentration of the catalyst, processing time) affect the obtained yield of the reducing sugars [43]. Determining the optimal pre-treatment conditions with respect to the yield of sugars is an important issue in the perspective of the cost-effectiveness of lignocellulosic biomass processing. In order to select the optimal conditions of the alkaline pre-treatment, based on the

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performed experiments according to the data from Table 4, the coefficients in the quadratic Equation (1) were determined. The Equation (1) was used for the estimation of the surface response area.

$$G_{eff} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 {X_1}^2 + \beta_6 {X_2}^2 + \beta_7 {X_3}^2 + \beta_8 {X_4}^2 + \beta_9 X_1 X_2 + \beta_{10} X_1 X_3 + \beta_{11} X_1 X_4 + \beta_{12} X_2 X_3 + \beta_{13} X_2 X_4 + \beta_{14} X_3 X_4 , \quad (1)$$

where β_i—coefficient in the quadratic equation.

In order to obtain a useful form of the mathematical model, given in Equation (1), the least significant variables were rejected in the consecutive iterative steps, i.e., for values of P above 0.05. The calculations revealed that the glucose yield (G_{eff}) can be estimated with a good accuracy ($R^2 = 0.798$) using the Equation (2):

$$G_{eff} = -1.1700 + 0.1275 X_1 + 0.0405 X_3 - 0.0126 {X_1}^2 - 0.0003 {X_3}^2 + 0.0076 X_1 X_4 - 0.0014 X_2 X_3 \quad (2)$$

Statistical parameters for the determined coefficients are presented in Table 6.

Table 6. Statistical parameters for the model coefficients according to the Box-Behnken design.

Coefficient	Estimated Value	Standard Deviation	*T*-Value	*P*-Value
β_0	−1.1700	0.26610	−4.397	0.00023
β_1	0.1275	0.04830	2.640	0.01490
β_3	0.0405	0.00916	4.420	0.00022
β_5	−0.0126	0.00567	−2.216	0.03732
β_7	−0.0003	0.00008	−3.525	0.00190
β_{11}	0.0076	0.00273	2.776	0.01102
β_{12}	0.0014	0.00036	−3.823	0.00093

The results of calculations, leading to the form of the model given by Equation (2) indicated that the least influence on the obtained glucose yield is described by: in the first power the pre-treatment time and the interaction of time and temperature, granulation and temperature, catalyst concentration and temperature as well as catalyst concentration and granulation; in the second power the time and granulation. The obtained experimental results are similar with the published results [44] presenting the parameters significantly affecting the results of alkaline pre-treatment with NaOH. However, the investigations were conducted for a different lignocellulosic material (rice straw).

2.3. The Effect of Alkaline Pre-Treatment on the Composition of Energetic Willow Hydrolyzates

In order to determine the effect of the alkaline pre-treatment on the change of biomass composition, the weight loss (biomass recovery), cellulose, hemicellulose and lignin content, lignin removal and cellulose and hemicellulose recovery rates were determined in the residues after the treatment (Table 7). It was found that an increase both of the catalyst concentration and of the temperature favors the weight loss of the processed material. The highest weight loss (40.19%) was observed for the process carried out at 80 °C and for NaOH concentration of 7% (*w*/*v*). The weight loss corresponds with the high removal rates of lignin (50.1%) and hemicellulose (73.2%).

The results of investigations indicate that it is not possible to completely remove lignin from the plant material as a result of alkaline treatment, even if high temperatures and high concentrations of alkaline reagents are used. Aggressive alkaline treatment of corncobs (160 °C, 10% NaOH *v*/*v*) removes approximately 85% of lignin [45]. The inability to completely remove lignin from the processed material may contribute to a reduced efficiency of enzymatic hydrolysis. This may be due to limited availability of sugar polymers for enzymes and the possibility of binding of active enzyme centers by lignin, which may lead to enzyme deactivation.

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Table 7. Changes in the biomass composition after alkaline pre-treatment.

No.	Cellulose Content (%)	Hemicellulose Content (%)	Lignin Content (%)	Biomass Recovery (%)	Lignin Removal (%)	Glucan Recovery (%)	Hemicellulose Recovery (%)
1	44.4	12.9	23.0	66.9	43.7	65.3	40.0
2	61.3	9.7	22.8	59.8	50.1	80.6	26.8
3	69.3	12.6	23.2	66.3	43.8	78.4	38.9
4	52.3	25.0	22.1	80.6	34.9	92.6	93.6
5	54.0	22.3	22.7	78.6	34.8	93.2	81.5
6	57.2	17.9	24.7	68.7	38.0	86.4	57.1
7	50.4	20.7	26.0	85.4	18.7	94.6	82.2
8	58.2	13.3	27.7	72.1	26.9	92.3	44.7
9	53.9	14.6	25.4	79.4	26.2	94.2	54.1
10	54.2	20.8	24.0	79.2	30.5	94.4	76.5
11	48.2	22.2	28.1	79.4	18.4	84.1	81.9
12	47.6	21.4	24.3	83.1	26.2	87.0	82.8
13	48.3	21.4	25.7	72.2	32.2	76.6	71.8
14	48.4	18.7	22.9	79.9	33.1	85.0	69.7
15	50.7	19.1	23.5	79.7	31.5	88.9	70.9
16	47.3	18.5	24.2	78.1	31.1	81.2	67.2
17	47.8	16.6	28.8	81.3	14.3	85.5	62.6
18	45.4	17.3	22.6	76.3	37.0	76.2	61.5
19	45.6	11.4	23.7	66.8	42.1	66.9	35.3
20	44.3	18.9	22.4	78.8	35.5	76.8	69.4
21	47.6	13.4	24.5	69.4	37.8	72.0	43.2
22	53.9	20.0	23.5	77.5	33.4	91.8	82.2
23	61.4	18.3	25.5	70.7	34.1	95.0	76.3
24	40.4	14.8	25.1	78.9	27.6	70.1	54.5
25	47.2	15.8	22.9	82.7	30.7	85.8	60.7
26	52.7	19.1	22.6	76.8	36.5	89.0	68.1
27	51.1	16.2	22.0	75.7	39.1	85.0	57.1
28	41.0	12.2	23.9	72.9	36.3	65.7	41.4
29	56.8	25.9	24.0	78.7	30.9	98.2	94.8

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The results of investigations (Tables 5 and 7) indicate that an important factor influencing the result of the alkaline pre-treatment is the degree of fragmentation (granulation) of the processed material. It was found that with an increase of the degree of fragmentation, the weight loss of the processed biomass increases, despite the lower temperature and shorter treatment time (compare experiments 9 and 23, Table 7).

It was observed that for mild alkaline pre-treatment, i.e., NaOH concentration below 4% and temperature below 40 °C, the loss of hemicellulose and lignin is insignificant, and the change in the chemical composition of the material is probably mainly due to dissolution of the extractives in solution. This is evidenced by the fact that in the case of energetic willow, after the alkaline pre-treatment, only small amounts of extractable compounds remain, i.e., non-structural biomass components. The above conclusion is consistent with the literature data suggesting the loss of biomass during the alkaline pre-treatment results mainly from the removal of lignin, extractives and hemicelluloses [45,46]. The increase in temperature and the increase in the concentration of the catalyst causes the increase in the removal of lignin and hemicelluloses [47]. As on effect of alkaline treatment, hemicellulose passes into solution in the form of both simple sugars (pentoses, hexoses) and sugar oligomers [48]. As a result, the total yield of simple sugars, which is used as a carbon source for fermentation leading to the production of biofuels, is reduced. Alkaline pre-treatment also breaks down ester bonds between lignin and hemicellulose, which results in an increase of the porosity and specific surface area of the material and consequently increases the availability of cellulose structures for cellulolytic enzymes [49].

2.4. Enzymatic Hydrolysis of Pre-Treated Energetic Willow

After completion of the alkaline pre-treatment (delignification), the pre-treated biomass samples are further treated with biochemical methods. Enzymatic hydrolysis is intended to release monosugars from crystalline cellulose and from the remains of hemicellulose. The efficiency of this step is crucial for the conversion of the biomass to the desired products because monosugars are the source of carbon for fermentative microorganisms [50]. The conversion of cellulose and hemicellulose is catalyzed by glucosidase and hemicellulases. Cellulose hydrolysis can be carried out using enzymes such as endoglucanases, which hydrolyse internal β-1,4-glycosidic bond, exoglucanases removing the end of a glucose chain monomers and dimers, and glycosidases, which hydrolyse dimers of glucose–cellobiose, considered as an inhibitory sugar for cellulolytic enzymes [51]. Degradation of cellulose is complicated if the microfibers are stabilized by internal and external hydrogen bonds, and they are surrounded by hemicellulose polysaccharides, i.e., mannans and xylans. Cellulase plays an important role as it catalyzes the degradation of cellulose into fermentable sugars. Cellulose hydrolysis begins with the adsorption of cellulase on the surface of the cellulose, followed by the degradation of cellulose to sugars and cellulase desorption from the surface of the biomass. Hemicellulose structure can be broken by various enzymes. Xylan is hydrolyzed under the action of endoxylanase and β-xylosidase, which cause its degradation to xylooligosaccharides. Enzymes such as α-glucuronidase, α-arabinofuranoside and acetyloxyl esterase cleave side groups and heterosilyl chains [52,53]. Glucomannan hydrolyzates are digested by β-mannanase and β-mannosidase. In addition to the quality of the hydrolytic enzyme, digestibility of cellulose and hemicellulose is influenced by such factors as pH, temperature, time, and porosity of the carrier, degree of crystallization and consistency of the cellulose [54].

The enzymatic hydrolyzates may be potentially used in fermentation processes, as a source of carbon, therefore it is crucial to remove the enzymes from the hydrolyzates after the process. This is why the enzyme immobilization was applied. Enzymes immobilized on diatomite may be separated by centrifugation following the hydrolysis [55]. Physical adsorption of enzymes requires soaking the diatomite in the enzyme solution [56]. Depending on the pH of the solution and the isoelectric point, the equilibrium of the immobilization may be affected. Therefore, to avoid non-linear changes, buffers such as Mc'Ilvainae or phosphate buffer must be used.

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The obtained values of glucose mass, sum of reducing sugars and glucose efficiency for the raw material subjected directly to enzymatic hydrolysis were respectively 130 mg/g biomass, 210.5 mg/g biomass and 0.12. The above given results of enzymatic hydrolysis were taken as a reference for further investigations.

The results indicate that for temperature below 55 °C and catalyst concentration below 1%, enzymatic hydrolysis of the raw material results in higher yields than the hydrolysis of the material after the alkaline treatment. This is probably due to the removal of non-structural sugars during the alkaline treatment in the above-mentioned conditions. With an increase of the concentration of alkaline catalyst above 4% and temperature above 55 °C, the concentration of sugars in the hydrolyzates after enzymatic hydrolysis increases. The highest value of glucose efficiency (0.79) from energetic willow was obtained for the catalyst concentration of 7%, temperature 55 °C and pre-treatment time 7 h. Blank hydrolysis test (i.e., without enzymes, with diatomite only) did not reveal the presence of simple sugars in the liquid.

The effect of the enzyme immobilization on the glucose efficiency was investigated. For this purpose, the alkaline pre-treated energetic willow (granulation 2 mm, catalyst concentration 4%, temperature 55 °C, treatment time 3.75 h) was subjected to enzymatic hydrolysis with free enzymes and with enzymes immobilized on diatomaceous earth. The yield of glucose after hydrolysis with free and immobilized enzymes was 0.67 and 0.46, respectively. Despite the lower glucose efficiency, it was decided to apply the method with immobilization, because it allows a quick separation of enzymes from hydrolyzates and enzymes recovery. The immobilization of cellulolytic enzymes on diatomaceous earth and their application on lignocellulosic materials is not widely described in the literature. These studies are a prelude to further work aimed at material processing and enzymatic hydrolysis in a semi-continuous or continuous manner. The possibility of reusing immobilized cellulolytic enzymes on diatomite for saccharification processes of lignocellulosic biomass may be an object of further experiments. In addition, it is believed that some components resulting from alkaline pre-treatment may be adsorbed on the surface of diatomaceous earth. The group of these components include, among others, phenolic derivatives such as furfural and 5-HMF. These compounds may inhibit the subsequent fermentation to biofules carried out on the obtained hydrolyzates. It may be a subject of further research in this field.

2.5. Influence of Alkaline Pre-Treatment Parameters on the Glucose Efficiency

Based on the regression Equation (2), Figures 1–4 are given, presenting the response of the glucose efficiency as a function of independent variables of the alkaline pre-treatment. Based on the analysis of the presented plots, the optimal process parameters for the alkaline pre-treatment were then proposed, taking into account the glucose efficiency and costs of the process.

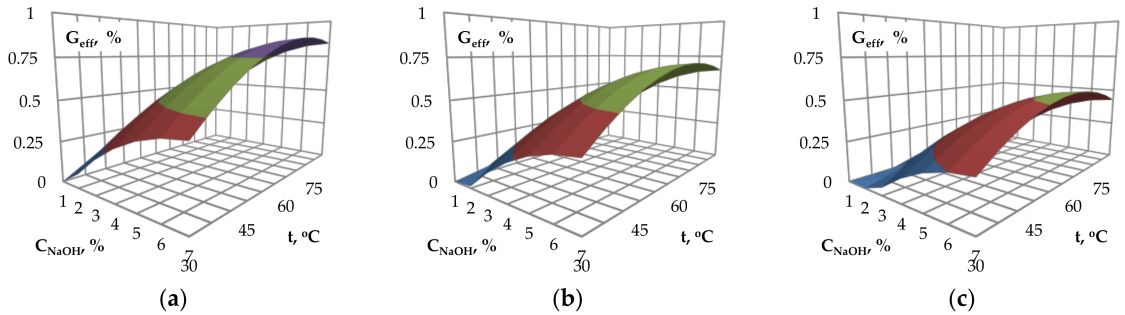

Figure 1. Influence of the catalyst concentration and the temperature on the glucose efficiency for the pre-treatment time of 7 h: (**a**) Granulation 0.25 mm; (**b**) Granulation 2 mm; (**c**) Granulation 4 mm.

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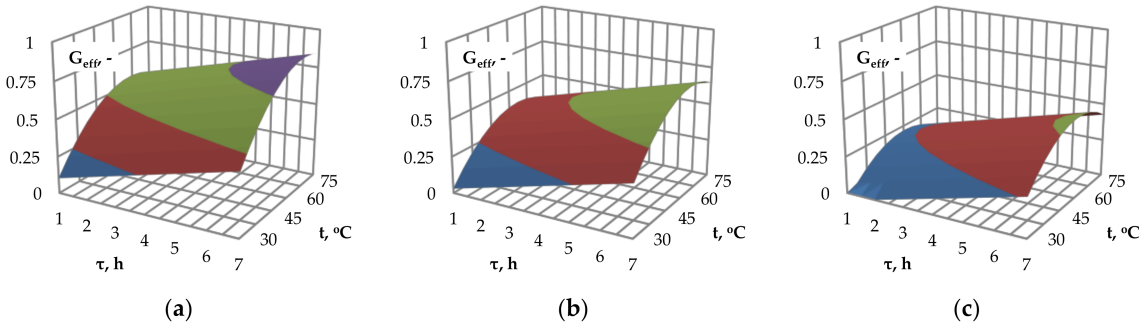

Figure 2. Influence of the pre-treatment time and temperature on the glucose efficiency for the catalyst (NaOH) concentration of 7%: (**a**) Granulation 0.25 mm; (**b**) Granulation 2 mm; (**c**) Granulation 4 mm.

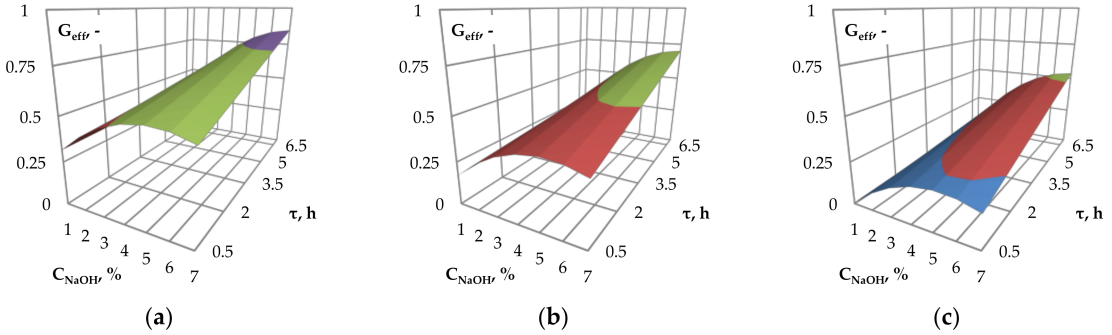

Figure 3. Influence of the catalyst concentration and pre-treatment time on the glucose efficiency for the pre-treatment temperature of 60 °C: (**a**) Granulation 0.25 mm; (**b**) Granulation 2 mm; (**c**) Granulation 4 mm.

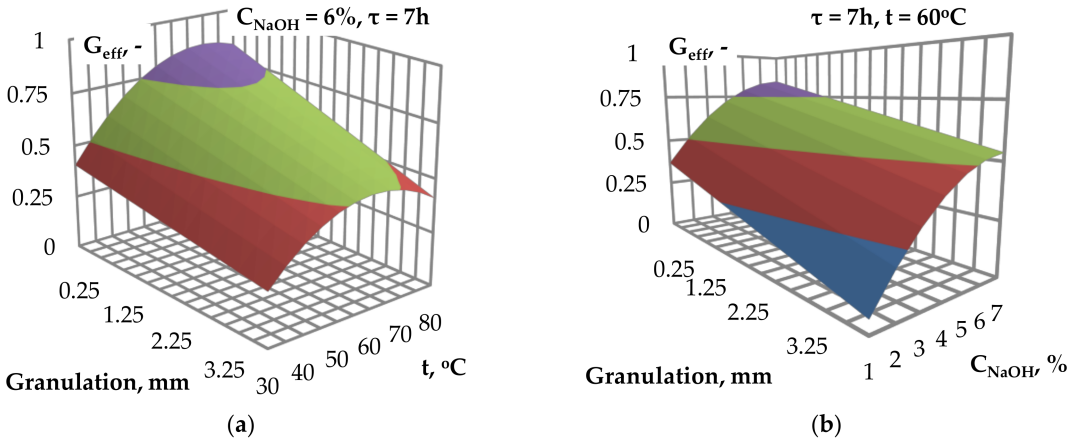

Figure 4. The surface response plots for the effect of granulation and temperature (**a**) and granulation and concentration (**b**) on the glucose efficiency.

Figure 1a–c presents the values of glucose efficiency as a function of NaOH catalyst concentration and temperature. The investigated process parameters influence the glucose efficiency and optimal values of process parameters may be selected. An increase in the catalyst concentration from 1% to approximately 6% results in an increase of glucose yield. For NaOH concentration above 6%, no further increase of glucose yield was observed.

The effect of temperature on the glucose yield is similar to the effect of catalyst concentration. An increase of the temperature to approximately 75 °C, 65 °C and 55 °C, respectively for granulation of

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0.25, 2 and 4 mm, results in an increase of glucose efficiency. The above-mentioned temperature values are followed by stabilization (Figure 1a) or slight reduction (Figure 1b,c) of the glucose efficiency.

Figure 2a–c show the influence of the pre-treatment time and temperature on the glucose efficiency. In the investigated range of alkaline pre-treatment time, an increase in the processing time results in an increase of glucose efficiency. This can be explained by the lack of excessive degradation of the material during processing in the investigated temperature range.

An influence of the catalyst concentration and the pre-treatment time on the glucose efficiency is presented in Figure 3a–c. The given response surface areas as a function of the mentioned process parameters suggest that optimal values of catalyst concentration for the highest glucose efficiency can be selected. As stated before, the highest glucose yield was obtained at NaOH concentration of approx. 6–6.5%. For concentrations above the mentioned value, degradation of the lignocellulosic material may proceed [45], resulting in no further increase in the concentration of reducing sugars in the hydrolyzate.

Figure 4a,b show the effect of granulation, temperature and catalyst concentration on the glucose efficiency. The decrease of the size of biomass particles result in an increase of glucose efficiency. This is due to an increase on the available materials surface area for the contact with catalyst and the improvements of mass and heat transfer along with the reduction of the biomass particle sizes during the alkaline pre-treatment [5,57,58]. Similarly as shown in Figures 1–3, in the studied range of process parameters, the response surface areas in Figure 4a,b indicate that the optimal value of catalyst concentration and the temperature of the alkaline pre-treatment can be selected for the highest glucose efficiency.

Based on the obtained model, the optimal conditions for the conversion of biomass to glucose are proposed as follows: granulation 0.25 mm, catalyst concentration 7%, temperature 80 °C, pre-treatment time 7 h. The given values of parameters correspond to the maximum values of glucose yield in the investigated range. The optimal conditions of alkaline pre-treatment of rice straw presented in the literature [44] taking into account temperature, NaOH concentration and pre-treatment time are 81.8 °C, 2.82% and 56.7 min, respectively. Application of these conditions allowed to obtain 252.62 g of glucose from 1 kg of rice straw. In other studies on the pre-treatment of the algae mixture *Tetraselmis suecica* and *Chlorella* sp. with sodium hydroxide [43], for a temperature of 120 °C, a concentration of NaOH 2% and a time of 30 min, 81 mg of reducing sugars from 1 g of dry biomass were obtained. This yield was doubled after enzymatic hydrolysis. The optimal pre-treatment conditions to maximize the glucose yield are obtained at 2.5% NaOH concentration, performing the process at 120 °C for 40 min. According to the initial content of glucose in the raw material, the best results corresponds to glucose production of 202 g from 1 kg of vineyard pruning [59].

2.6. Optimization of the Alkaline Pre-Treatment

The obtained results show that the parameters of the alkaline pre-treatment, such as the temperature, the catalyst concentration, the pre-treatment time and the size of hydrolyzed material particles have a significant influence on the result of enzymatic hydrolysis and thus on the yield of monosugars. According to the authors of this work, the selection of conditions for alkaline pre-treatment requires optimization not only with respect to the efficiency of glucose, but also due to the costs of treatment (Table 8). This approach is particularly justified when scaling-up, because often a small increase in the efficiency is accompanied by an enormous increase in the costs of process realization.

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Table 8. Cost assessment of alkaline pre-treatment.

Parameter	Substance, Device or Power Requirement	Pre-Treatment Effect, Parameter Value or Quantity	Assessed Cost per 100 g of Biomass (€) [1]
Granulation	Garden shredder, 2400 W	Primary fragmentation–chips	0.04
	Ultracentrifugal mill, 1300 W	Granulation: 4 mm	0.02
		2 mm	0.02
		0.25 mm	0.08
Catalyst concentration	1 N NaOH	1 ÷ 7%	0.01 ÷ 0.02
Temperature	Thermostated shaker, 1500 W	30 ÷ 80 °C	0.01 ÷ 0.12
Pre-treatment time		0.5 ÷ 7.5	0.01 ÷ 0.1

[1] Assumptions for calulations: The cost of electricity 1 kWh = 0.16 €; the cost of 1 kg of 1 N NaOH = 3 €; the experimentally determined working time of devices was assumed so as to obtain 100 g of pre-treated lignocellulosic biomass; the experimentally determined working time of the shaker related to maintaining the desired temperature was assumed; labor costs and other depreciation costs were excluded.

An exemplary economic analysis of the energetic willow alkaline pre-treatment shows that fragmentation, pre-treatment time and temperature significantly affect the costs of the process. Therefore, in order to reduce the costs of biomass processing, the optimal values of the process parameters should be lower than the maximum values from the investigated range. Therefore, it seems reasonable to compare the efficiency and relative increase in glucose efficiency for the process parameters under examination. Figures 5 and 6 show the above-mentioned dependencies calculated on the basis of the proposed model. This approach makes it possible to indicate ranges of process parameters, above which the efficiency increase is disproportionately small compared to the increase of the process costs. Cost assessment analysis is not common in published articles.

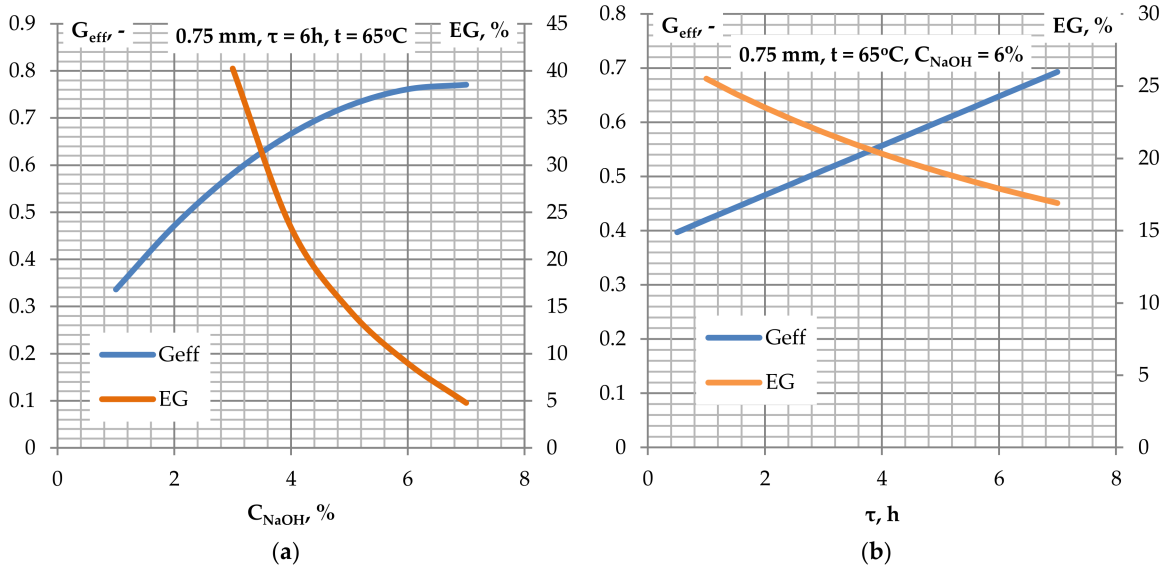

Figure 5. Effect of catalyst concentration (**a**) and pre-treatment time (**b**) on the glucose efficiency and relative glucose efficiency gain.

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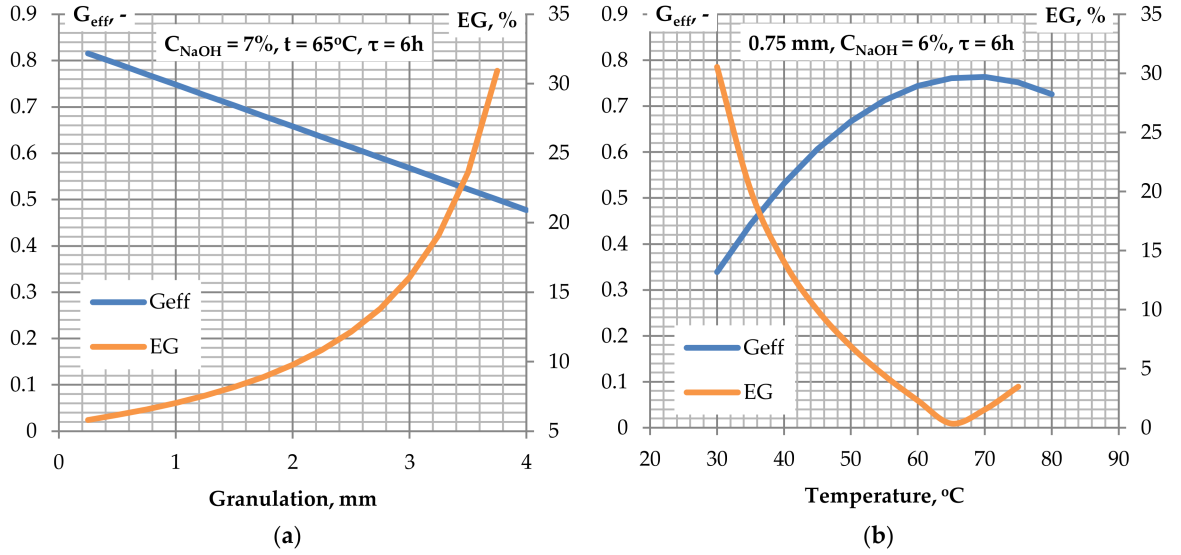

Figure 6. Effect of granulation (**a**) and pre-treatment temperature (**b**) on the glucose efficiency and realative glucose efficiency gain.

Figure 5a shows the effect of NaOH concentration on the efficiency and the efficiency gain of glucose. For NaOH concentrations above 6%, the glucose yield is practically unchanged (EG gain <5% when C_{NaOH} increases from 6% to 7%). Because of the small proportion of sodium hydroxide cost in the total cost, it is proposed to use C_{NaOH} = 7%.

Figure 5b shows the effect of the pre-treatment time on the glucose efficiency and relative glucose efficiency gain. The increase in the duration time of the alkaline treatment results in an increase in glucose efficiency. Considering the costs related to the pre-treatment time due to the need to maintain the selected temperature during the alkaline treatment, as well as decreasing values of the relative glucose efficiency gain, a duration time of 6 h is suggested as suitable for the pre-treatment.

Figure 6a presents the influence of granulation on the glucose efficiency and the relativeglucose efficiency gain as a result of alkaline treatment. The highest glucose yields were obtained for granulation of 0.25 mm. However, the costs of obtaining the indicated degree of material fragmentation are relatively high. Because the efficiency gains attain the lowest values for granulation below 1 mm, keeping high values of glucose efficiency, granulation of 0.75 mm are selected to be optimal. The indicated value is economically justified, because the technical conditions of the milling process allow to obtain particles of the indicated size at the same costs as for 2 mm granulation. Material grinding to a size of 0.25 mm requires two-stage grinding, during which additional local overheating of the material and possible thermal degradation may take place.

Figure 6b shows the influence of the temperature on the glucose efficiency and relative glucose efficiency gain. The dependencies show that the highest efficiency value was obtained for a temperature of approximately 65 °C, which corresponds with the minimum on the relative glucose efficiency gain curve. The indicated temperature value is optimal for alkaline pre-treatment of biomass from *Salix viminalis* L.

The results presented in Figures 1–6 and economic analysis (Table 8) for the optimal conversion of biomass from *Salix viminalis* L. indicate that the following process parameters were optimal: granulation 0.75 mm, NaOH concentration 7%, pre-treatment time 6 h, temperature 65 °C. Enzymatic hydrolysis of the material previously treated with alkali in the above-mentioned conditions gave a glucose yield and a sum of reducing sugars of 415.7 g and 487 g respectively, from 1 kg of biomass, the values correspond to the glucose efficiency of 0.797 calculated from the proposed model. For comparison, the corn stover treatment with 10% sodium hydroxide at a temperature of 140 °C and for 30 min, it was possible to obtain a concentration of reducing sugars of 440 mg/g biomass [60]. Significantly milder conditions of

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grass pre-treatment (C_{NaOH} = 0.5%, 60 °C and t = 0.5 h) but ultrasounds-assisted, allowed to obtain approximately 260 mg/g of reducing sugars [61]. Optimal alkaline treatment conditions proposed in this paper allow to obtain up to twice the performance of reducing sugars. However, it should be noted that the type of raw material used has a significant influence on the final result of glucose efficiency.

2.7. Influence of the Material Type on the Glucose Efficiency

The effect of the chemical composition of lignocellulosic biomass on the efficiency of bioconversion for six selected raw materials: three wood (energy willow, energetic poplar and beech) and three agricultural waste (field grass, wheat straw and corncobs) was compared. In the first series of experiments, the material was subjected to enzymatic hydrolysis, while in the second series—the alkaline treatment was first carried out, followed by the enzymatic hydrolysis. The raw materials used for the research were fragmented in a mill using a 0.75 mm sieve. The two-stage conversion process was carried out under the optimal conditions indicated above, considering the economic analysis of the process (i.e., C_{NaOH} = 7%, temperature 65 °C, pre-treatment time 6 h). The yield of glucose for wood raw materials after enzymatic hydrolysis was 230, 234 and 324 mg from 1 g of biomass for beech, energetic willow and energetic poplar, respectively. The two-stage process allowed to increase the glucose efficiency by approximately 1.4 times for poplar and 1.8 times for willow and beech. A comparable increase in the glucose release was obtained after hydrolysis of corncob, i.e., 1.65. The largest increase in the biomass to glucose conversion (approximately 3 times) was obtained for grass and straw.

The obtained results of glucose efficiency are similar to other literature data [62], e.g., for alkaline pre-treatment of corncobs (dilute NH_3, temperature 75 °C, time 96 h). Authors of the paper [48]obtained a yield of reducing sugars of 850 mg from 1 g of wheat straw after the alkaline treatment (temperature 121 °C, time 30 min, C_{NaOH} = 2%). The efficiency of reducing sugars under optimal conditions proposed in the present work for grass is 1.34 times higher than for the process carried out at 60 °C, in 30 min and for C_{NaOH} = 0.5%. Despite a significant improvement in the yield of reducing sugars due to the use of alkaline treatment, it occurred that the yield of monosugars obtained by direct enzymatic hydrolysis of the raw material is decent. Depending on the type of raw material subjected to hydrolysis, after 16 hours of hydrolysis, the yield of reducing sugars was obtained in the range of 38.5% for straw to 82% for energetic poplar. Such high yields of glucose yield from untreated material can be the result of enzyme immobilization on diatomite. For comparison, using non-immobilized cellulolytic enzymes ensured the yield of glucose in the level of 17% for barley straw hydrolysis, when compared to the theoretical values [8,63,64] and only 3.6% of glucose for switch grass [65,66]. Untreated rice straw hydrolysis for 72 h allowed to obtain only 20% of total glucose [66]. Also, the direct enzymatic hydrolysis of sugarcane bagasse allowed to obtain only 13% of reducing sugars [67].

The highest weight loss of biomass was noted for agricultural waste materials, which may be connected to their chemical composition that differs from the composition of woody lignocellulosic materials, such as beech, poplar or willow. Grasses and grains contain significant amounts of non-structural ingredients, including dyes and minerals that can easily pass into the catalyst solution. As a result, there is a significant change in their chemical composition after the pre-treatment, which results in a higher biomass loss than for trees. Additionally, the highest degree of lignin removal was also obtained for the waste agricultural materials. Significant impact on the removal of lignin from a low—lignin content material, such as agricultural waste, using alkaline pre-treatment, is confirmed in the available literature [65]. It is different in the case of the acid treatment, which depending on the conditions, allows for even 100% removal of hemicelluloses. However, its effect on the lignin content is smaller [66]. Acid treatment with 85% phosphoric acid at 50 °C for 5 h allowed to remove 88.5% and 91.3% hemicellulose from the straw and maize straw, respectively. However, the loss of lignin was 15.1% for maize straw and zero for maize [67]. The use of sulfuric acid to treat wheat straw allowed for the hydrolysis of 96% hemicellulose [68].

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Changes in the structure of biomass resulting from alkaline and/or enzymatic treatment are presented on SEM images (Table A2). The Appendix B presents SEM images for raw materials (Table A2, Figures a) and after selected pre-treatment mode: alkaline pre-treatment (Table A1, Figures b), enzymatic hydrolysis of raw material (Table A2, Figures c) and after a two-stage treatment i.e., alkaline pre-treatment and enzymatic (Table A2, Figures d). Images of biomass samples after the alkaline pre-treatment revealed the loosening of materials resulting in the increased availability of cellulose and hemicellulose for hydrolytic enzymes. This is probably mainly due to the removal of lignin during the alkaline pre-treatment. SEM images taken for materials after enzymatic hydrolysis and after two-stage treatment indicated that the material degradation occurs at these stages. Also visible in the images are the characteristic structures of diatomaceous earth, which are the remains of immobilized enzymes. As can be seen from the data presented in Table 9, the cumulative yield of simple sugars after enzymatic hydrolysis from the raw material is higher for the material after the alkaline treatment. The degree of lignin removal is also higher for these processes. This demonstrates the desirability of converting biomass to simple sugars in a two-stage process.

Table 9. Composition of enzymatic hydrolyzates obtained from raw (R) and alkaline pre-treated (H) biomass.

Parameter		Energetic Willow	Beech	Energetic Poplar	Triticale	Meadow Grass	Corn Cobs	Unit
Glucose	R	234.0	230.0	324.0	150.6	141.8	258.0	$mg/g_{biomass}$
	H	415.7	418.0	443.0	453.7	414.0	428.0	
Xylose	R	20.9	25.0	42.0	9.7	8.0	21.8	$mg/g_{biomass}$
	H	39.6	39.6	42.6	30.3	31.3	32.9	
Galactose	R	17.8	19.6	17.0	0.0	5.0	24.5	$mg/g_{biomass}$
	H	24.7	20.8	17.0	15.3	6.3	29.2	
Mannose, arabinose	R	15.6	17.6	12.0	13.5	15.4	42.7	$mg/g_{biomass}$
	H	7.0	9.4	12.6	9.9	41.8	11.2	
Lignin	R	29.4	26.3	26.3	22.4	16.8	14.1	%
	H	23.9	23.6	21.6	8.6	4.1	0.7	
Biomass recovery		64.0	53.4	62.5	38.0	24.3	23.8	%
Lignin removal		47.8	52.1	48.6	85.4	94.1	98.8	

2.8. Influence of the Alkaline Pre-Treatment on Inhibitory Compounds Formations

During the alkaline pre-treatment of lignocellulosic biomass, delignification results in the formation of lignin derivatives, mainly phenolic compounds. During acidic hydrolysis furan derivatives, including furan and 5-HMF and acids, including acetic, levulinic and acetic acids, are formed depending on the conditions. HMF and furfural are formed during the dehydration reaction of simple sugars catalyzed by acids [69,70]. Most of the by-products of pre-treatment are formed due to the decomposition of hemicellulose. Usually, an increase in their concentration is related to the decrease in the concentration of simple sugars derived from hemicellulose hydrolysis [71,72]. The type and concentration of formed inhibitors depend on the processing conditions. The influences of temperature (140 °C and 160 °C) and the concentration of sulfuric acid (5–20 g/dm^3) on the formation of inhibitors were examined. It turned out that the higher the temperature and the higher the concentration of sulfuric acid, the higher the concentration of acetic and formic acids, HMF and furfural, with the highest concentration observed for formic acid [69,73]. Some of the inhibitory compounds may be adsorbed on the surface of the wood sediments. It is possible to subject such a sediment to enzymatic hydrolysis and then perform the detoxification [74–77]. It is possible to rinse the sediment several times with, for example, water [44], for elution of the inhibitors. The effect of using sludge water on the effectiveness of inhibitor removal was investigated. It turned out that six rinsing cycles allows to remove the total content of 87% formic acid, 64% acetic acid, 86% furfural and 87% HMF [73].

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The addition of acetone to water for the purpose of elution from the inhibitor sludge, allowed to obtain a total removal of inhibitors from the sludge. Acetone dissolves organic compounds much more easily and thus removes them more effectively from the sediment. In addition, acetone can be easily recovered for being reused by vacuum distillation. The use of efficient washing of the sludge after the treatment avoids the need for its detoxification. Another situation occurs when the acid is used for direct hydrolysis of biomass. Then, it may be necessary to use the detoxification process [71,78].

The effect of the alkaline pre-treatment carried out under the optimal conditions in the presence of inhibitors in hydrolyzates was also examined. It turned out that a typical inhibitor present in the investigated hydrolyzates is acetic acidIts concentration after enzymatic hydrolysis prior and after the alkaline pre-treatment varied and was equal to 32.1 and 3.1 $mg/g_{biomass}$ for grass, 10.4 and 7.7 $mg/g_{biomass}$ for wheat, 8.7 and 3.0 $mg/g_{biomass}$ for corn cobs, 3.7 and 2.34 $mg/g_{biomass}$ for poplar, 11.0 and 3.35 $mg/g_{biomass}$ for willow and 10.0 and 6.7 $mg/g_{biomass}$ for beech. As can be seen, the effect of the pre-treatment has a significant impact on the concentration of acetic acid in the hydrolyzates. Similar observations were obtained as a result of another work concerning the alkaline pre-treatment catalyzed with NaOH, KOH and $Ca(OH)_2$ followed by enzymatic hydrolysis. It turned out that acetic acid was present in small concentrations only [77].

The direct application of enzymatic hydrolysis forthe lignocellulosic material does not result in the passage of the vast majority of known inhibitors to the hydrolyzate solution. When cellulolytic enzymes were applied on untreated rice straw supported with ultrasounds and surfactant, it turned out that only acetic acid was present in the hydrolyzate solution [77].

3. Materials and Methods

3.1. Chemical Reagents and Enzymes

- Chemical reagents (sodium hydroxide, sulfuric acid, hydrochloric acid, calcium oxide) are purity grade purchased from Chempur,(Piekary Śląskie, Poland)
- Chemical standards (Celobiose, Glucose, Mannose, Arabinose, Xylose, Galactose, Furfural, HMF, acetic acid, vanilic acid, vaniline,) with purity for analysis grade purchased from Sigma Aldrich (St. Louis, MO, USA)
- Chemicals for HPLC (Acetonityle, sulfuricacid) are HPLC grade purchased from Merck (Kenilworth, NJ, USA)
- Demineralized water for HPLC anayslis was filtered through a 0.45 µm filter
- Viscozyme L from *Aspergillus* sp. Novozyme Corp. (Davis, CA, USA) (Sigma Aldrich) enzyme complex containing a great variety of carbohydrosides: arabinase, cellulase, β-glucanase, hemicellulase, and xylanase T. opt. 35–40 °C pH 5–6.5
- Glucosidase $\geq$ 750 U/g from *Aspergillus* sp. Novozyme Corp. (Sigma Aldrich) T. opt. 35–45 °C pH 4–6.5
- Cellulase $\geq$ 800 U/g from *Aspergillus niger*, Novozyme Corp. (Sigma Aldrich) T. opt. 30–40 °C pH 4–5.8
- Cellulase $\geq$ 700 U/g from *Trichoderma reesei* ATCC 26921 Novozyme Corp. (Sigma Aldrich) T. opt. 35–40 °C pH 4–6.8
- Diatomite was purchased from Chempur
- Phosphate Buffer 0.1 M pH 5.7–8.0 (13.9 g NaH_2PO_4; 35.85 g $Na_2HPO_4 \times 12H_2O$, 500 mL water)
- Mc'Ilvainae's Buffer, pH 4.5 (44.1 mL; 0.02 M Na_2HPO_4; 55.9 mL 0.01 M citric acid and filled up to 1000 mL with distilled water)

3.2. Biomass Preparation

- The investigated lignocellulosic biomass materials were: energetic willow *Salix viminalis* L., energetic poplar *Populisindustria*, beech *Fagus* L., wheat straw *Triticum paleas*, corncobs *Zeamays*,

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meadow grass *Poapratensis*. Above given materials were collected from energetic plantations near Wejherowo, in northern part of Poland. The biomass of various age of stands were collected in summer 2016 and the investigations reported here were performed in autumn 2017, The air-dried lignocellulosic biomass was pre-ground with a garden shredder (Meec Tools, Skara, Sweden) to chops of approximately 3 cm in length and 1 cm in width and then ground on a Ultra Centrifugal Mill ZM 200 (RETCH, Haan, Germany). During grinding, sieves with a 0.25 mm, 0.75 mm, 2 mm and 4 mm diameter mesh were used. The material after grinding was stored in sealed containers at room temperature. Prior to the alkaline pre-treatment, the material was dried in a laboratory dryer at 105 °C for 4 h and then stored in a desiccator with a drying substance (NaOH). The material was subjected to enzymatic or alkaline pre-treatment and enzymatic hydrolysis.

3.3. Alkaline Pre-Treatment

- The lignocellulose biomass alkaline pre-treatment was carried out using sodium hydroxide. The reactions were carried out in 100 mL sealed vessels, in which 3 g of ground and dry lignocellulosic biomass and 60 mL of catalyst solution were placed. The vessels were placed for a definite time in a thermostated shaker. The reaction mixture was then neutralized with a hydrochloric acid solution, placed in centrifuge tubes, centrifuged and filtered through a Buchner funnel to separate the precipitate from the solution. The precipitate was then washed three times with water and twice with acetone. The resulting precipitate was dried for 24 h at 105 °C and placed in a desiccator. The alkaline treatment was carried out according to the Box-Behnken design (Tables 4 and 5).

3.4. Enzymatic Hydrolysis

- High quality enzyme preparations for the hydrolysis of lignocellulosic residues were used. According to the manufacturer's declaration the supplementation of enzyme mixtures with an additional portion of β-glucosidase may increase the hydrolysis yield to monosaccharides, due to the fact, that cellobiose is an inhibitor of cellulolytic enzymes [79]. Immobilized enzyme preparation requires placing 20 mL enzymatic solution (Viscozyme L, Glucosidase 0.85:0.15 m/m) in a 50 mL beaker with 2.5 g dry diatomite and stirring the solution for 1 h at low speed on a magnetic stirrer at room temperature. The diatomite with the immobilized enzyme must be washed in a column with a small amount (approximately 10 mL) of Mc'Ilvaine's buffer. After a few minutes the bed stabilizes. Next, the diluted diatomite must be stored under a layer of buffer (approximately 5 mm). To use the enzymatic hydrolyzing preparations, the bed should be shaken and then introduced to the solid residue of lignocellulosic biomass solution after basic hydrolysis.
- 0.2 g of milled and minced lignocellulosic biomass pre-treated with alkaline hydrolysis 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 shaker at 42 °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 were analyzed. Control experiments were carried without the addition of lignocellulosic material.

3.5. SEM Images and EDX Measurements

- Samples of lignocellulosic biomass were dried and gold-covered by sputtering method prior to analysis (sputter coater Quorum Q150TE, Quorum Technologies Ltd, Lewes, UK). SEM images were performed using SEM Zeiss EVO-40 (Carl Zeiss Microscopy GmbH, Jena, Germany). Energy dispersive X-ray spectroscopic measurements (EDX measurements) to reveal the elemental composition of investigated materials were carried out using EDS Bruker AXS Quantax 200 (Billerica, MA, USA). The EDX measurements were carried out on biomass materials without gold sputtering.

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3.6. Methods

- Data analysis and other calculations were performed using RStudio Desktop (v. 1.0.143) software.
- Total solids, ash and extractives of raw corn stover were determined according to the National Renewable Energy Laboratory (NREL) analytical procedure [62,80]
- The composition and analysis of sugar and lignin content in the native material and after basic hydrolysis were determined using the method described in the NREL procedure [62]. The content of cellulose and hemicellulose was determined by HPLC using a Rezex Pb^{2+} column (300 × 7.8 mm, 8 um) and a refractometric detector (Knauer). Water was used as the eluent, flow rate (0.6 mL/min).
- The composition and sugar content after enzymatic hydrolysis was determined by HPLC using a Rezex Pb^{2+} column (300 × 7.8 mm, 8 um) and a refractometric detector (Knauer, Berlin, Germany). Water was used as the eluent, flow rate (0.6 mL min).
- The presence and concentration of fermentation inhibitors was determined using HPLC with RID and UV-DAD detector and Shodex SH1011 column (300 × 7.8 mm, 8 um). Water with 5 mM $H_2SO_4$was used as eluent, flow rate 0.6 mL/min. Biomass recovery (BR) was calculated according to Equation (3):

$$\text{BR}(\%) = (\text{Pretreated biomass (g)}/\text{Original biomass (g)}) \times 100\% \quad (3)$$

- The glucan and xylan recovery was calculated from Equation (4):

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

- The lignin removal (LR) was calculated from Equation (5):

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

  where C—lignin content in (wt %).
- Glucose efficiency (G_{eff}) was calculated from Equation (6):

$$G_{\text{eff}} = M_{\text{glucose in hydrolzate}}/M_{\text{glucan in pretreated}}, \quad (6)$$

  where M—mass (g).
- Relative glucose efficiency gain (EG) was calculated as follows (7):

$$\text{EG} = |1 - (G_{\text{effi}}/G_{\text{eff i}-1})| \times 100\%, \quad (7)$$

  where i—value of G_{eff} for ith modeled point.

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Conflicts of Interest: The authors declare no conflict of interest.

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Appendix A

Table A1. Results of EDX measurements for elemental composition of investigated materials.

Element	wt %	Error %	Element	wt %	Error %
1st Set of Measurements			**2nd Set of Measurements**		
Energetic Willow					
C	51.809	5.884	C	52.432	5.938
O	47.625	5.612	O	47.132	5.555
Mg	0.110	0.033	Ca	0.213	0.033
Ca	0.176	0.032	Al	0.061	0.029
Al	0.106	0.031	Na	0.047	0.005
K	0.082	0.029	Mg	0.043	0.004
P	0.031	0.002	K	0.038	0.002
Si	0.025	0.002	Si	0.021	0.001
Cu	0.033	0.003	P	0.012	0.001
Energetic Poplar					
C	52.096	5.906	C	52.463	5.934
O	47.312	5.572	O	47.174	5.546
Na	0.108	0.035	Al	0.105	0.032
Al	0.097	0.031	Mg	0.088	0.028
Ca	0.137	0.031	Ca	0.058	0.002
Mg	0.077	0.031	K	0.031	0.003
K	0.068	0.029	Cu	0.041	0.002
Cu	0.071	0.031	Si	0.017	0.001
Si	0.016	0.001	P	0.018	0.001
P	0.015	0.001			
Meadow Grass					
C	47.557	5.703	C	42.241	5.166
O	44.250	5.462	O	48.695	5.871
Si	2.087	0.118	Si	4.386	0.216
K	2.818	0.114	K	2.167	0.094
Ca	1.029	0.058	Cl	1.332	0.073
Cl	0.828	0.044	Al	0.215	0.038
P	0.416	0.037	Ca	0.315	0.037
S	0.271	0.038	Mg	0.181	0.038
Mg	0.179	0.039	S	0.152	0.033
Cu	0.246	0.032	P	0.110	0.031
Fe	0.215	0.035	Cu	0.199	0.038
Al	0.099	0.032			
Triticale					
C	45.663	5.357	C	48.421	5.654
O	49.499	5.846	O	47.985	5.768
Si	3.014	0.157	Si	0.863	0.065
K	0.795	0.052	K	1.170	0.063
Mg	0.199	0.039	Ca	0.867	0.053
Ca	0.288	0.036	Mg	0.119	0.034
Al	0.125	0.033	S	0.120	0.032
P	0.130	0.032	Cl	0.124	0.031
S	0.126	0.031	Al	0.069	0.030
Cu	0.094	0.033	Cu	0.159	0.035
Fe	0.049	0.004	P	0.075	0.029
Ti	0.012	0.001	Fe	0.021	0.002

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Table A1. *Cont.*

Element	wt %	Error %	Element	wt %	Error %
1st Set of Measurements			**2nd Set of Measurements**		
Beech					
C	53.008	6.019	C	52.667	5.983
O	46.031	5.457	O	46.510	5.551
K	0.210	0.033	Ca	0.289	0.036
Mg	0.112	0.033	K	0.148	0.032
P	0.134	0.032	Na	0.079	0.033
Cu	0.138	0.031	Al	0.067	0.030
Al	0.101	0.031	Mg	0.058	0.030
Si	0.063	0.029	Cu	0.076	0.031
S	0.052	0.028	P	0.035	0.003
Cu	0.093	0.032	S	0.032	0.002
Cl	0.034	0.002	Cl	0.033	0.002
Corncobs					
C	48.426	5.699	C	46.728	5.564
O	45.410	5.494	O	46.040	5.673
Al	1.690	0.109	K	4.581	0.167
Si	1.741	0.102	Cl	1.013	0.062
K	1.497	0.073	Fl	0.440	0.177
Fl	0.479	0.175	Ca	0.459	0.042
Cl	0.206	0.034	Si	0.179	0.035
Cu	0.249	0.039	Mg	0.150	0.036
Ca	0.122	0.031	Al	0.118	0.033
P	0.075	0.029	Cu	0.201	0.038
			P	0.086	0.030

Appendix B

Table A2. SEM images of investigated lignocellulosic materials: (**a**) Raw material; (**b**) Material after alkaline pre-treatment; (**c**) Material after enzymatic hydrolysis; (**d**) Alkaline pre-treated and enzymatically hydrolyzed material.

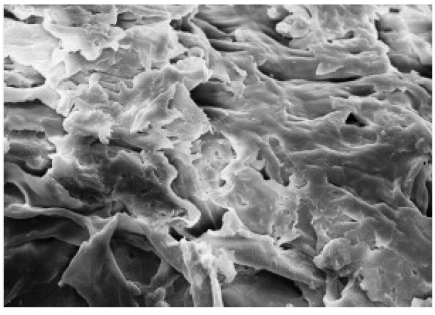
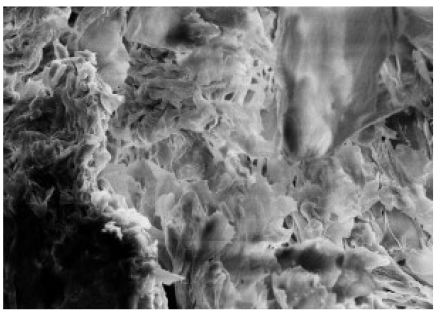
Corn Cobs	
	
(**a**)	(**b**)

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Table A2. *Cont.*

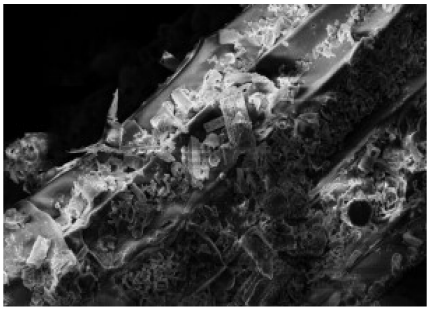
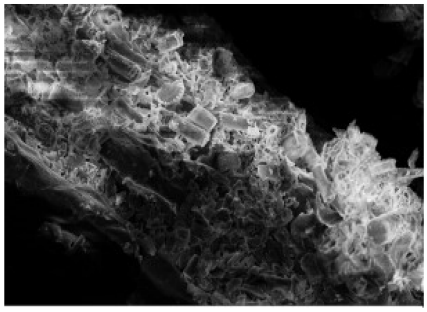
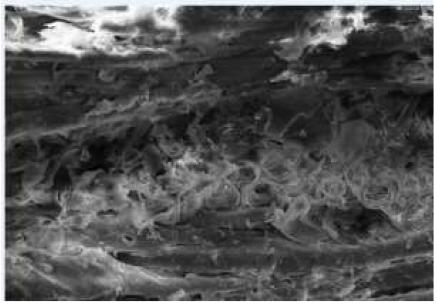
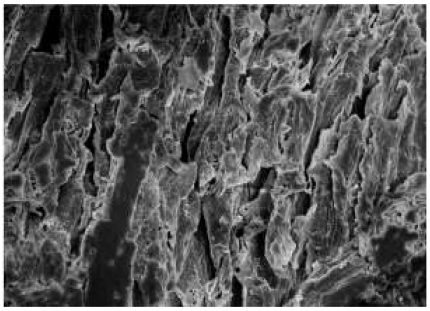
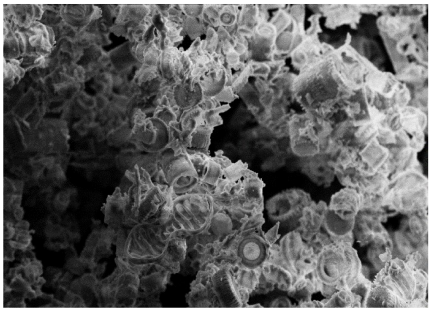
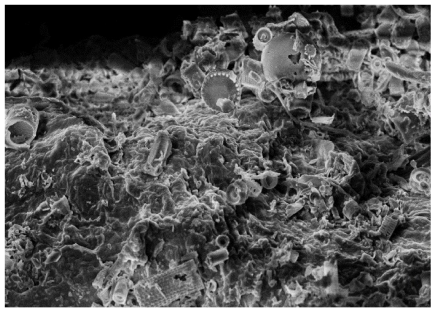
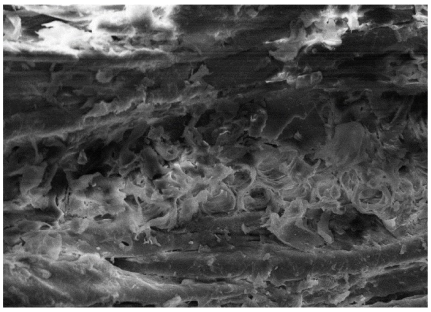
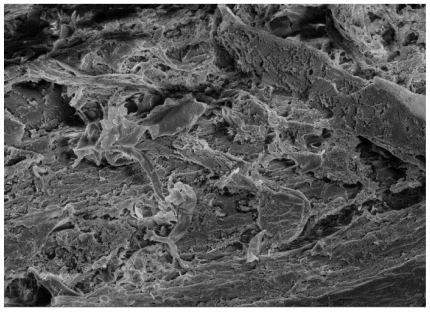
	
(**c**)	(**d**)
Beech	
	
(**a**)	(**b**)
	
(**c**)	(**d**)
Energetic Poplar	
	
(**a**)	(**b**)

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Table A2. *Cont.*

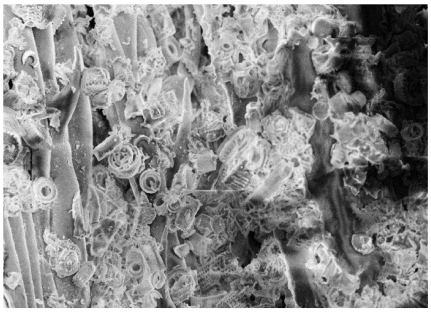
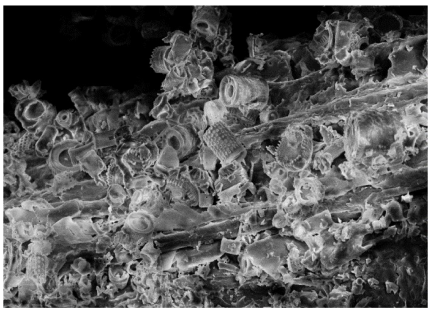
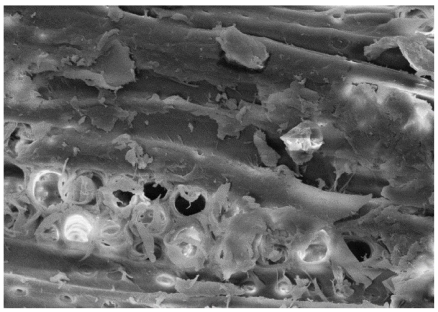
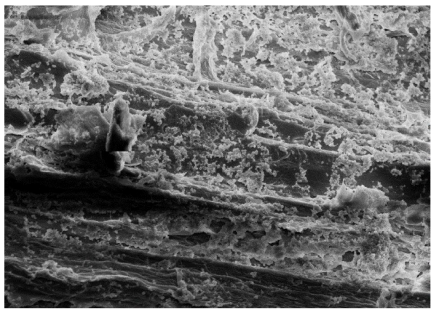
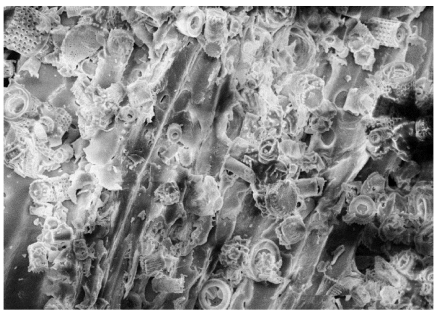
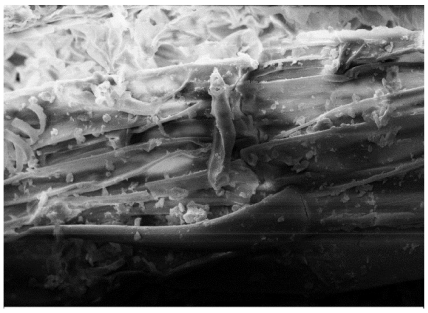
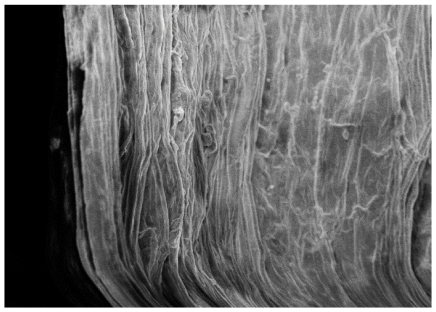
	
(**c**)	(**d**)
Energetic Willow	
	
(**a**)	(**b**)
	
(**c**)	(**d**)
Meadow Grass	
	
(**a**)	(**b**)

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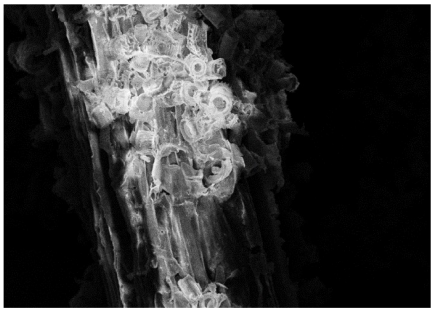
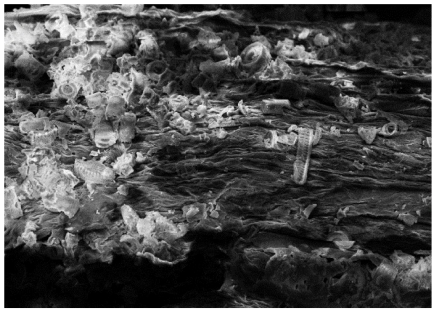
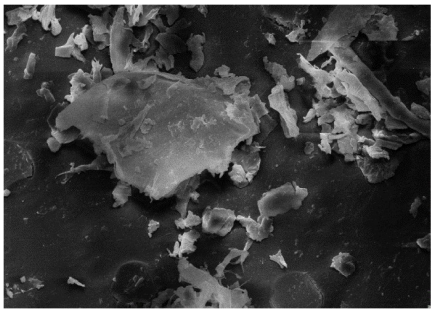
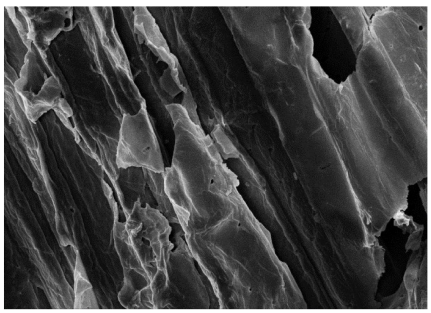
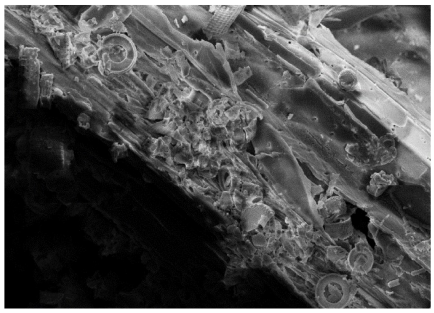
Table A2. *Cont.*

	
(**c**)	(**d**)
Triticale	
	
(**a**)	(**b**)
	
(**c**)	(**d**)

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