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CONDITIONS OF LIGNIN, HEXOSANS AND PENTOSANS DETERMINATION IN SELECTED LIGNOCELLULOSIC MATERIALS

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Pentosans and hexosans contents were determined in lignocellulosic materials by the o-toluidine colorimetric method based on hydrolysis of lignocelluloses with 72% H_2SO_4 in optimal conditions followed by determinations of sugars in acidic hydrolyzates.

The basic components of lignocellulosic materials are hexosans (mainly cellulose), pentosans and lignin. The methods of determining these compounds may be physical (e.g. employing infrared spectroscopy [1]) or chemical, the latter dividing into the direct and indirect as far as lignin determination is concerned. The direct methods consist in acid hydrolysis of the material which decomposes into monosaccharides and other water-soluble byproducts while lignin remains insoluble and can be determined by weighing. Saccharides may be determined spectrophotometrically or chromatographically [2-5]. In indirect methods, lignin is removed from the material into the solution and the non-lignin substances are determined by weighing. Most of these methods are labour- and time-consuming and efforts are made to discover fast and accurate methods of determining components of lignocellulosic materials. Today many researchers opt for the former group of methods using hydrochloric or sulfuric acid of various concentration as the carbohydrates-hydrolyzing agent, various hydrolysis times and different substrate-to-acid ratios [6-8].

One of the more popular methods of lignin and carbohydrates determination in lignocellulosic materials is that described by Effland [9] consisting in hydrolysis of the carbohydrates in these materials to monosaccharides with 72% H_2SO_4 and determination of lignin by weighing. However, treatment with such concentrated sulfuric acid leads to the formation of many byproducts which often lead to inflated figures for lignin and deceptively low figures for carbohydrates. In this research we attempted to check whether the conditions of carbohydrates hydrolysis proposed by Effland are optimal for determinations of lignin, pentosans and hexosans in selected lignocellulosic materials. The

materials we used were much more susceptible to enzymatic and acid hydrolysis than the wood and wood pulp used in experiments described by Effland [9]. Straw is not yet used in biotechnology as energy and carbon source for microorganisms, but many research centers are looking into possibilities of using in just this role.

METHODS

A) SUBSTRATES

Two lignocellulosic materials — rape and wheat straw — were used in our experiments, each being pretreated differently.

The straw was ground in an impact grinder and the fractions passing through a sieve of 0.43 mm gauge were used for further processing. In the first stage the wheat straw was autoclavesteamed at 200°C for 10 min following Targoński's procedure [11]. In the second stage after autoclave steaming, the straw was again heated (135°C for 45 min) with 1% NaOH water solution (5:1 solution-to-straw volume ratio), washed with water until alkaline reaction disappeared in the filtrate, and finally dried at 120°C to constant mass. This treatment prepares straw for enzymatic hydrolysis which is not the subject of this report.

B) LIGNIN DETERMINATION

To determine optimal conditions of wheat and rape straw hydrolysis we performed experiments with:

— different concentrations of 72% sulfuric acid (2.5 and 3 cm^3 of 72% H_2SO_4 per 250 mg pretreated straw);
— different times of hydrolysis at 30°C (30 and 60 min);
— different times of autoclave hydrolysis at 120°C (20, 40 and 60 min).

250 mg samples of substrates prepared in the manner described above placed in tightly-stoppered Erlenmeyer flasks and treated with 2.5 or 3.0 cm^3 of 72% H_2SO_4. The straw was thoroughly soaked in the acid solution and placed on a shaker in a 30°C water bath for 30 or 60 min. The samples were than diluted with distilled water (28 cm^3 for every cm^3 of acid) and further hydrolyzed at 120°C in an autoclave for 20, 40 or 60 min. The hot solution was filtered through a previously dried Schott G-3 glass filter and then washed with distilled water till the disappearance of acid in the filtrate. The filters together with the residue were dried to constant mass at 105°C, and lignin content was determined by subtracting ash.

C) DETERMINATION OF PENTOSANS AND HEXOSANS

The amounts of pentoses and hexoses as hydrolysis products were determined in the filtrate deprived of lignin using o-toluidine [12]. The hydrolyzate was

neutralized with 30% NaOH, its volume measured, 0.5 cm³ samples transferred to stoppered test tubes, treated with 4 cm³ of o-toluidine reagent, and heated in a water bath for 30 min. After cooling, absorbance was determined in the samples at 385 and 630 nm wavelength with respect to control. The content of pentoses and hexoses was calculated by the determinants method after first determining the absorbance of the standard glucose and xylose solution:

$$C_p = \frac{\begin{vmatrix} A_{385} & A_2 \\ B_{630} & B_2 \end{vmatrix}}{\begin{vmatrix} A_1 & A_2 \\ B_1 & B_2 \end{vmatrix}} \text{ (mg/cm3)}$$

$$C_h = \frac{\begin{vmatrix} A_1 & A_{385} \\ B_1 & B_{630} \end{vmatrix}}{\begin{vmatrix} A_1 & A_2 \\ B_1 & B_2 \end{vmatrix}} \text{ (mg/cm3)}$$

where A_{385} and B_{630} are absorbances of the studied solutions at 385 and 630 nm, A_1 and B_1 — absorbances of standard xylose solution of 1 mg/cm³ concentration, and A_2 and B_2 — absorbances of standard glucose solution of 1 mg/cm³ concentration.

Amounts of pentosans and hexosans were calculated using conversion coefficients 0.88 and 0.9.

DISCUSSION OF RESULTS

Optimal conditions of hydrolysis of carbohydrates in the lignocellulosic substrate were found by determining the regression function in a planned experiment and search along the function's gradient [5]. Three parameters were optimized in the first stage:

x_1 — amount of 72% H_2SO_4used in hydrolysis,
x_2 — time of hydrolysis with 72% H_2SO_4,
x_3 — time of autoclave hydrolysis at 120°C in diluted H_2SO_4.

The optimal parameters were assumed to be as follows:

x_1 — 2.75 cm³ with variable interval = 0.25 cm³,
x_2 — 45 min with variable interval = 15 min,
x_3 — 40 min with variable interval = 20 min.

A matrix was compiled on the basis of the above data (Table 1) and the pentoses and hexoses contents were determined in the studied samples with o-toluidine. Regression coefficients were determined taking total saccharides content into account:

— for rape straw $Y = 0.511 + 0.021x_1 + 0.06x_2 - 0.061x_3$,
— for autohydrolyzed wheat straw $Y = 0.584 - 0.034x_1 + 0.029x_2 - 0.072x_3$,
— for twice pretreated wheat straw $Y = 0.72 + 0.053x_1 + 0.095x_2 - 0.023x_3$.

Table 1. Scheme of experimental selection of optimal conditions of carbohydrates determination in lignocellulosic materials

Factors	Amount of 72% H_2SO_4 (ml)	Time hydrolysis		Amounts of carbohydrates (mg/g)		
		conc. H_2So_4 (min)	dil. H_2SO_4 (min)			
Basic level	2.75	45	40			
Variable interval	0.25	15	20			
Higher level	3.00	60	60			
Lower level	2.50	30	20			
Code symbols	x_1	x_2	x_3	Y_1	Y_2	Y_3
a) variables	—	—	—	481	417	678
	+	—	—	418	688	448
	—	+	—	580	909	662
	+	+	—	709	983	668
	—	—	+	417	668	523
	+	—	+	483	728	426
	—	+	+	481	673	466.5
	+	+	+	520	693	491

Y_1 — Amounts (mg) of carbohydrates after hydrolysis of 1 gram of rape straw.
Y_2 — Amounts (mg) of carbohydrates after hydrolysis of 1 gram of autohydrolysed wheat straw.
Y_3 — Amounts (mg) of carbohydrates after hydrolysis of 1 gram of twice pretreated wheat straw.

Further optimization of conditions of lignocellulosic materials hydrolysis was done by searching along the gradient. To do this, Tables 2-4 were compiled and the composition of the studied lignocellulosic materials determined on the basis of specific hydrolysis parameters (Table 5).

We assumed as optimal hydrolysis conditions those for which reducing sugars (total pentosans and hexosans) figures were the highest. The amounts of 72% sulfuric acid used to hydrolyze 250 mg of steamed straw ranged from 2.6 to 2.8 cm^3. In the case of wheat straw subjected to the alkaline treatment this amount had to be greater — 3.15 cm^3, one of the reasons for this being that the specific volume of such straw is greater than that of untreated straw and more acid is needed to soak it.

Regardless of the kind of straw and its pretreatment, the optimal time of hydrolysis of its carbohydrates was 50 min. However, in the case of repeated hydrolysis with diluted sulfuric acid at 120°C, this optimal time was much shorter, namely 30 min as opposed to the 60 min recommended by Effland's original method.

Table 2. Optimization of rape straw hydrolysis using the linear approximation gradient

Variables	Basic level x_i°	Variable interval $x_{\ }$	Regression coefficient $b_i \times 10$	$10b_i\Delta x_i$	$\Delta x_i^\circ + 10b_i\Delta x_i$	$\Delta x_i^\circ + 20b_i\Delta x_i$	$\Delta x_i^\circ + 30b_i\Delta x_i$
x_1 (ml)	2.75	0.25	0.2	0.05	2.8	2.85	2.9
x_2 (min)	45	15	0.6	9.0	54	63	72
x_3 (min)	40	20	–0.6	–12.0	28	16	7
Amounts:							
Carbohydrates	(mg/g) 583				590	510	462
Lignin	(mg/g) 176				180	180	180

Table 3. Optimization of hydrolysis of pretreated wheat straw using the linear approximation gradient

Variables x_i	Basic level x_i°	Variable interval Δx_i	Regression coefficient $b_i \times 10$	$10b_i\Delta x_i$	$\Delta x_i^\circ + 10b_i\Delta x_i$	$\Delta x_i^\circ + 20b_i\Delta x_i$	$\Delta x_i^\circ + 30b_i\Delta x_i$
x_1 (cm^3)	2.75	0.25	0.53	0.13	2.88	3.01	3.14
x_2 (min)	45	15	0.95	13.5	58.5	72	85.5
x_3 (min)	40	20	–0.23	–4.6	35.4	30.8	26.2
Amounts:							
Carbohydrates	(mg/g) 650				674	727	741
Lignin	(mg/g) 300				312	300	318

Table 4. Optimization of hydrolysis of twice pretreated wheat straw using the linear approximation gradient

Variables x_i	Basic level x_i°	Variable interval Δx_i	Regression coefficient $b_i \times 10$	$10b_i\Delta x_i$	$\Delta x_i^\circ + 10b_i\Delta x_i$	$\Delta x_i^\circ + 20b_i\Delta x_i$
x_1 (cm^3)	2.75	0.25	–0.34	–0.085	2.7	2.6
x_2 (min)	45	15	0.29	43.5	49.3	53.6
x_3 (min)	40	20	–0.72	–14.4	25.6	11.2
Amounts of Carbohydrates	(mg/g) 610.5				715	585

As can be seen in Table 2 and 3, the determined amounts of lignin were similar, this being evidence that the conditions optimal for carbohydrates hydrolysis were favourable for lignin content determination. The contents of lignin, hexosans and pentosans obtained in straw hydrolysis under conditions which we found to be optimal are given in Table 5.

Table 5. Lignin, hexosans and pentosans contents in rape straw and pretreated wheat straw

Substrate	Lignin (%)	Hexosan (%)	Pentosan (%)
Rape straw	17.6	32.1	20.6
Autohydrolysed wheat straw	31.6	51.5	12.9
Twice pretreated wheat straw	31.8	60.0	6.7

CONCLUSION

1. The proposed method of determining lignin, pentosans and hexsosans in wheat and rape straw ensures rapid results in any laboratory.
2. It was demonstrated that the selection of optimal hydrolysis conditions for each of the studied lignocellulosic materials in crucial for correct determinations of pentoses and hexoses in the hydrolyzate.

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WARUNKI OZNACZENIA LIGNINY, HEKSOZANÓW I PENTOZANÓW W WYBRANYCH SUROWCACH LIGNOCELULOZOWYCH

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Streszczenie

Przeprowadzono optymalizację hydrolizy węglowodanów za pomocą 72% H_2SO_4 w celu oznaczenia ligniny, pentozanów i heksozanów w materiałach lignocelulozowych o różnej podatności na enzymatyczną hydrolizę, tj. słomie rzepakowej oraz wstępnie przygotowanej dwiema metodami — słomie pszennej. Substrat hydrolizowano w temp. 30°C stosując zmienny moduł kwasu do substratu różny czas hydrolizy stężonym, a następnie rozcieńczonym kwasem siarkowym. Nierozpuszczalną pozostałość oznaczono wagowo, a po odjęciu zawartości popiołu otrzymywano zawartość ligniny w substracie. W hydrolizatach oznaczono ilość pentoz i heksoz z odczynnikiem o-tuluidynowym. Optymalne warunki hydrolizy określono wyznaczając funkcje regresji za pomocą eksperymentu planowego, wykonanego metodą całkowitego doświadczenia czynnikowego oraz poszukiwań po wynikającym z niej gradiencie.

Stwierdzono, że optymalne warunki hydrolizy 72% H_2SO_4 były różne dla różnych substratów, co wykazano na podstawie ilości oznaczonych węglowodanów. Czas hydrolizy, niezależnie od substratu, wynosił 50 min, a optymalne jego ilości uzależnione były od rodzaju surowca poddawanego hydrolizie. Natomiast najkorzystniejszy czas hydrolizy w autoklawie wynosił 20-30 min i był krótszy niż w oryginalnej metodzie oznaczania ligniny wg Efflanda. Optymalne warunki dla hydrolizy węglowodanów były również optymalne dla oznaczania ligniny.