STUDY ON THE MODIFICATIONS IN THE COMPOSITION OF SUGARS FROM LIGNOCELLULOSIC SUBSTRATES EXPOSED TO THE ACTION OF FUNGI

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The paper presents the results concerning the modifications in the sugar composition resulted from lignocellulosic substrates subjected to the action of lignolytic fungi: *Pleurotus ostreatus* and *Phanerochaete chrysosporium.* The substrates used were formed by various mixtures of corn cobs and oak tree leaves, oak tree sawdust and oak tree leaves, and oak tree bark – oak tree leaves, all in ratios of 2:1 (marked as mixtures I, II and III). The evaluation of the sugar composition of the substrates biodegraded by the above-mentioned fungi, for 30 days, was performed after hydrolysis, using 1N sulfuric acid at 120 °C, for one hour, followed by sodium carbonate treatment until a pH of 5.8 was reached. The quantitative determinations were performed through gas chromatography. The gathered data showed that the highest degree of the heteropolysaccharide degradation corresponded to *Pleurotus ostreatus*, while the fungus *Phanerochaete chrysosporium* cultivated under the same conditions degraded carbohydrates at a lower level. Also, a large consumption of pentoses was recorded, evaluated through the variation of the xylose amounts, which rapidly decreased in case of the *Pleurotus ostreatus* microorganism, in comparison with the substrate inoculated by *Phanerochaete chrysosporium*.
Similarly, the hexoses consumption levels (mainly of glucose) were quite modest in the case of *Pleurotus ostreatus* and quite significant for *Phanerochaete chrysosporium*.

Keywords: lignocellulosic substrates, lignolytic fungi, carbohydrate degradation

INTRODUCTION

The penetration of lignolytic fungi into the rigid, structural edifice of the vegetal cell walls takes place in distinct, complex phases of cleaving the bonds between carbohydrates and lignin, through the action of enzymatic systems, which releases phenolic compounds that are later on degraded via redox reactions.[1-5]

Located mainly inside the vegetal cells, at the interface between cellulose and lignin, hemicelluloses are made of heteroside chains, either linear or branched, with a low degree of polymerization (50 to 200 carbohydrate units) and are the initial target of the fungal attack, when it comes to the degradation of lignocellulosic materials.[4]

At the same time, it was recorded that distinct fungal strains consume preferentially certain sugars, therefore modifying in the end the ratios between the carbohydrates of the initial substrate.[3,4]

Knowing these aspects is most important for the optimization of the biotechnological processing of vegetal biomass, which can allow selective applications of various fungal strains, according to their potential of enzymatic cleavage of lignocellulosic residues.[2,4-7]

The paper evaluates the structural units of carbohydrates existent in the initial substrates and after precisely delimited periods of fungal attack, using gas chromatography. Therefore, the objectives were as follows:

- the identification of the consumption degree for the hemicellulosic sugars of the oak tree sawdust, the oak tree bark and corn cobs after the fungal attack, using certain strains selected at the Institute of Food Chemistry, Bucharest;
- the evaluation of carbohydrates consumption by the fungi *Pleurotus ostreatus* and *Phanerochaete chrysosporium*;
- the implementation of analytical methods that allow the characterization of the modifications that occurred in the composition of the carbohydrates, in the lignocellulosic substrates submitted to fungal attack.

EXPERIMENTAL

Substrates

The action of some lignolytic fungi was closely recorded, exclusively regarding natural substrates, with no chemical reagents added in order to create a carbon:nitrogen ratio of 25:1-35:1, considered as optimum for the composting processes. As the corn cobs, the oak tree sawdust and bark contain low levels of nitrogen, mixtures with oak tree leaves were used, which are rich in nitrogen. The oak tree sawdust and bark were provided by Foresta Arges S.A.

Microorganisms

The fungi used for each of the mixtures were *Pleurotus ostreatus* (*PO*) and *Phanerochaete chrysosporium* (*PC*), obtained from the collection of the Institute of Food Chemistry, Bucharest (ICA), strains 3164 and, respectively, 3141.

Preparation of substrates

Before fungi inoculation, a sterilization of the mixtures had been done, so as to avoid the influence of saprophyte microorganisms from the starter substrates.

The lignocellulosic materials, the corn cobs, the oak tree sawdust and the oak tree bark, as well as the oak tree leaves – as nitrogen source, were dried to constant mass and chopped to particles of sizes ranging between 0.1 and 0.5 cm. The following experimental mixtures were prepared:

I. Corn cobs – oak tree leaves (ratio 2:1)
II. Oak tree sawdust – oak tree leaves (ratio 2:1)
III. Oak tree bark – oak tree leaves (ratio 2:1).

They contained a carbon:nitrogen ratio between 1:25-1:35.

From the chopped, dried and uniform materials, 25 g samples were collected from each substrate, and were moistened with 75 mL of water in glass beakers, which were later sterilized in an autoclave for one hour at 121 °C.

Inoculation and incubation

After cooling, the samples were inoculated with 1 cm^3 of the experimental fungal cultures developed onto the Petri dishes. The solid medium in which the lignolytic fungi were maintained, in testing tubes, was the Bacto – Potato – Dextrozo – Agar medium (39 g/L), provided by the DIFCO laboratories. Glass bottles, sealed with cotton corks, which allow only micro-aeration, were incubated for one month at 37 °C.

Hydrolysis

After incubation, the free carbohydrates were extracted with 80% aqueous ethanolic solutions, while the remaining residue was submitted to hydrolysis using 1N sulfuric acid at 120 °C. Later, the hydrolysis products were treated with calcium carbonate until a pH of 5.8 was reached and further purified with 0.3% activated carbon until 0 transparency.

After removing the calcium sulfate precipitate, the clear extract was used to determine the contents of the remaining carbohydrates in substrates after the fungal attack. The differences between the hemicellulosic carbohydrates in the initial substrate and after the fungal attack, for every mixture, according to the inoculated fungal strain, allowed estimating not only the global consumption rate of hemicellulosic sugars, but also the preferential consumption for some sugars.[4,7-9]

The preference for hydrolytic cleaving, using 1N sulfuric acid is given by the fact that, for this concentration, the sugars cleaved mainly result from non-cellulosic heteroglycans, while the lignocellulosic structure remains basically unaffected, and the hydrolysis reagent is easily removed by a precipitation reaction.

Determining the α and β anomers for each of the carbohydrates was performed through high resolution gas chromatography (HRGC), using a FISONS apparatus, series 8000, equipped with a FID detector, at 250 °C.

Separating individual monosaccharides was performed using their derivatives obtained through silanization with hexamethyl disilazane. The trimethylsilyl derivatives (TMS), in their α and β forms, were obtained.[6,9,10]

The TMS derivatives were further extracted with heptanes, as they were separated by a capillary column with an OV_1 support and with a column length of 25 m x 0.32 mm internal diameter.

The thermal processing consisted in a submission for 3 minutes to a temperature of 150 °C, for another 3 minutes to 220 °C and additionally, another 2 minutes to 220 °C. The internal standard was inositol. The detection limit for individual carbohydrates was of 20 nanograms for each of the components.

The global solubilization level of the aromatic compounds was evaluated through UV absorption spectrometry, using a VARIAN spectrometer (S.U.A.), at wavelengths between 200 and 300 nm.

The carbohydrates content in the initial substrate is presented in Table 1 and Fig. 2a, while their levels after the incubation process, when using lignolytic fungi, are shown in Table 2 and Fig. 2b.

We have also determined, for the three substrates, the contents of free and of total carbohydrates (as shown in Tables 3 and 4).

The remaining solid residue resulted after the solid fraction removal was conditioned by protective ventilation with warm air. It was evaluated comparing with the initial level of biomass subjected to hydrolysis.

The average yield of the remaining residue was situated between 58% and 65%.

This residue might be successfully used as natural fertilizer.

RESULTS AND DISCUSSION

The initial level of monoses corresponding to the heteropolysaccharides existent in the initial mixture (e.g. mixtures I and II) are presented in Table 1. The remaining carbohydrates levels are presented in Table 2.

Table 1
Compared areas and retention times for standard and experimental substrates and the proportion of carbohydrates

Carbohydrate anomer	Retention time, R.T.	Standard	Mixture I		Mixture II	
			Compared area	Carbohydrate content (%)	Compared area	Carbohydrate content (%)
α-arabinose	7.28	309.431	184.333	1.19	64.339	0.42
β-arabinose	7.90	403.820	249.885	1.22	83.500	0.41
α-xylose	9.36	359.773	2259.474	12.40	400.337	2.20
β-xylose	10.78	476.165	2887.369	12.10	546.209	2.30
α-galactose	13.46	312.051	46.902	0.30	61.007	0.39
α-glucose	14.18	426.059	521.311	2.40	580.295	2.70
β-galactose	14.61	473.488	97.324	0.41	553.738	2.32
β-glucose	16.71	414.531 687.632	687.632	3.30	738.661	3.55

Table 2
Heteropolysaccharides levels of solid residue of the lignocellulosic materials after the fungal attack

Component carbohydrates and their anomers % of dry substance	Mixture I inoculated with		Mixture II inoculated with	
	P.O.	*P.C.*	*P.O.*	*P.C.*
α-arabinose	0.35	0.09	0.21	0.14
β-arabinose	0.25	0.40	0.48	0.51
α-xylose	2.76	2.37	1.80	1.72
β-xylose	2.84	2.24	1.59	1.62
α-galactose	0.44	0.36	0.22	0.28
α-glucose	2.04	0.88	1.38	0.27
β-galactose	1.55	1.22	0.21	0.32
β-glucose	3.14	0.63	0.56	0.45

P.O. – Pleurotus ostreatus; *P.C. – Phanerochete chrysosporium*

In Figure 1, the chromatogram obtained for a standard mixture of carbohydrates, using high-resolution gas chromatography (HRGC) is presented. The lower limit of determination of 0.1 g/kg (representing 20 ng for each of the carbohydrates, injected from the final derivative extract) from which a peak of 10 mm height at a noise of 1 mm was obtained. Figures 2a and 2b show chromatograms of monosaccharides obtained for substrate I (mixture of corncobs and

dry oak leaves), without hydrolysis (a) and with hydrolysis (b).

The content of free and total carbohydrates in the three substrates was determined (as shown by Tables 3 and 4). For all experimental versions, total consumption rate is higher in the case of *Pleurotus ostreatus*. For mixture I, total level of the heteropolysaccharides shows a diminishing of 33.23%, for mixture II of 54.2% and for mixture III of 49.03%.

The total rate of consumption in the case of *Phanerochaete chrysosporium* considering mixture I was of 14.03%, for mixture II of 8.85% and for mixture III of 15.62%. When it comes to its preferences towards certain carbohydrates, the preferential consumption of *Pleurotus ostreatus* was recorded for certain pentoses, mostly for xylose. Thus, in the case of mixture I, the xylose level decreased after 30 days of incubation, from 201.1 g/kg to only 112.0 g/kg. Mixture II showed the same trend, as the xylose levels decreased from 165.4 g/kg to 68.0 g/kg, while the decrease in mixture III was from 51.7 g/kg to 30.5 g/kg.

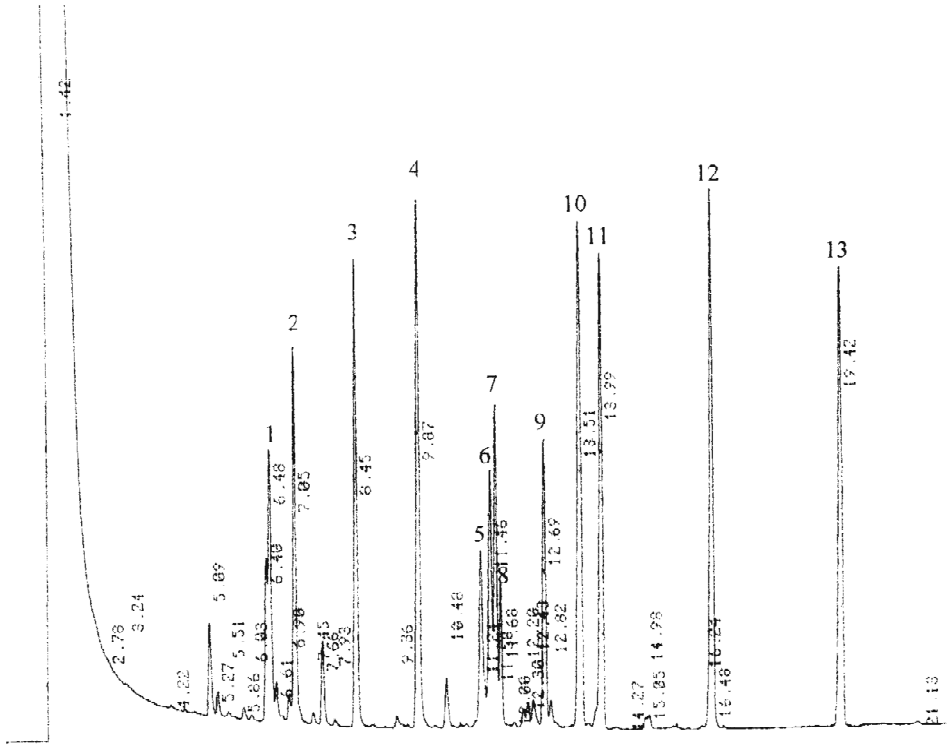


Figure 1: Chromatogram of a standard mixture of carbohydrates: l. α-arabinose, 2. β-arabinose, 3. α-xylose, 4. β-xylose, 5.6.7.8 fructose, 9. α-galactose, 10. α-glucose, 11. β-galactose, 12. β-glucose, 13. inositol

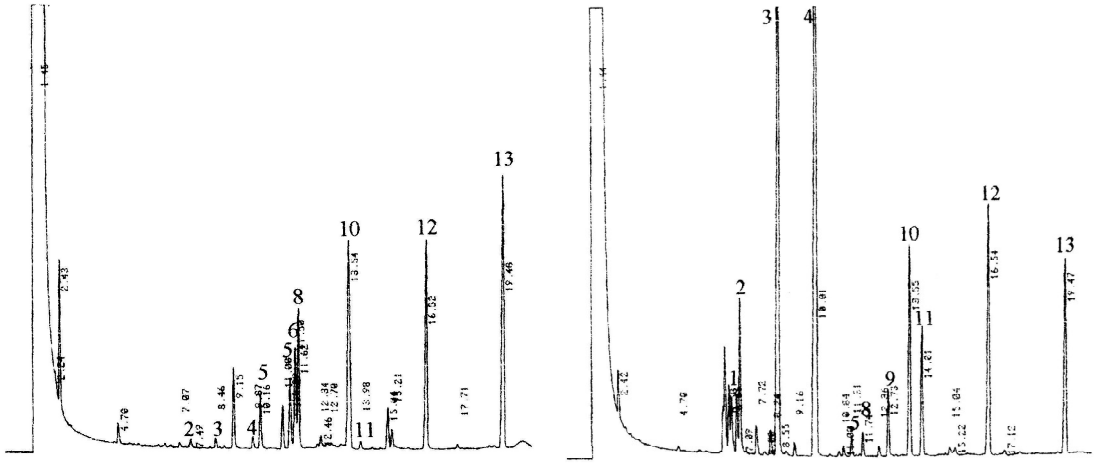
Table 3
Level of carbohydrates (CHD) (g/kg) in the initial substrates

Compound	Substrate I		Substrate II		Substrate III	
	Free CHD	Total CHD	Free CHD	Total CHD	Free CHD	Total CHD
Arabinose	0.17	97.80	0.07	33.20	0.28	38.20
Xylose	0.35	201.10	0.05	165.40	0.04	51.70
Fructose	6.08	4.60	2.02	8.90	0.37	3.60
Galactose	0.31	26.80	0.36	25.70	0.11	17.70
Glucose	7.30	43.60	3.53	26.30	1.39	13.60
Total	14.21	374.90	6.03	259.70	2.19	124.80

Table 4
Level of carbohydrates (g/kg) after incubation (30 days) with lignolytic fungi

Compound	Substrate l		Substrate II		Substrate III	
	P.O	*P.C*	*P.O*	*P.C*	*P.O*	*P.C*
Arabinose	22.4	73.6	1.3	28.3	4.5	32.2
Xylose	112.0	200.6	68.0	163.8	30.5	54.4
Fructose	1.5	4.0	6.8	8.8	3.8	1.8
Galactose	24.6	19.8	17.1	23.1	10.9	20.8
Glucose	34.6	11.6	28.4	12.7	13.9	4.1
Total	253.3	329.4	121.6	236.7	63.6	114.3

P.O. – Pleurotus ostreatus; *P.C. – Phanerochete chrysosporium*



a) b)

Figure 2: Chromatograms of carbohydrates in substrate I (mixture of corncobs and dry oak leaves in a ratio of 2:1); a) without hydrolysis and; b) with hydrolysis

Less significant was the decrease in the hexose content under the action of *Pleurotus ostreatus*. Therefore, in the case of mixture I, the glucose content presented a decrease from 43.6 g/kg to only 34.6 g/kg, while in the case of mixture II, it decreased from 26.3 g/kg to 25.4 g/kg, and for mixture III from 13.6 g/kg to 4.1 g/kg. In the case of *Phanerochaete chrysosporium*, the decrease of the xylose levels was practically insignificant. For example, in mixture I a decrease occurred, from 201.1 g/kg to only 200.6 g/kg, while in mixture II, the decrease was from 165.4 g/kg to 163.8 g/kg, and in mixture III – from 51.7 g/kg to 50.4 g/kg. It seems thus that pentoses are less preferred by *Phanerochaete chrysosporium*. On the other hand, the decrease in the hexose amount was more obvious, as in the case of mixture I the decrease was from 43.6 g/kg to 11.6 g/ kg, while for mixture II it was from 26.3 g/kg to 12.7 g/kg and mixture III – from 13.6 g/kg to 4.1 g/kg.

CONCLUSION

The GC of TMS derivatives proved to be an effective method to measure the level of carbohydrates in the lignocellulosic substrates. It is essential to maintain anhydrous conditions during the derivatisation step, since the derivatisation reagent reacts with hydroxyl groups. The above described GC method could be used with enough precision in order to depict the lignocellulosic materials. The comparative analysis of the experimental data shows that the degradation of heteropolysaccharides is greater for the treatment with *Pleurotus ostreatus*, in opposition to the treatment with *Phanerochaete chrysosporium* – for substrate I, the degradation is about four times greater, for substrate II it is 6 times greater, while for substrate III, about 3 times larger. For all analyzed substrates, we have shown that *Pleurotus ostreatus* preferentially consumes pentoses and *Phanerochaete*

chrysosporium mainly consumes hexoses. The gathered experimental data are confirmed by their natural habitat: *Pleurotus ostreatus* prefers xylose, as it is mainly found in forests, on tree barks, rich in pentoses, while *Phanerochaete chrysosporium* is found in soils in which the organic compounds might be mostly the result of cereal residues, therefore their consumption is preferentially based on hexoses, mainly glucose, thus the impact on the beta glucosidic bonds existing in cellulosic structures.

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