”Babeş-Bolyai” University Cluj-Napoca

Faculty of Chemistry and Chemical Engineering

RESEARCH REGARDING THE OBTAINING OF THE SECOND GENERATION BIOFUELS FROM LIGNOCELLULOSIC BIOMASS

PhD Thesis Summary

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(as in the full text)

EXPERIMENTAL PART

Keywords: wood waste, cellulose, carbohydrates, gas chromatography, delignification, hydrolysis, glucose, bioethanol

INTRODUCTION

PhD thesis addresses a current topic that is development of modern technologies for obtaining the second generation biofuel by superior capitalization of lignocellulosic biomass.

PhD thesis presents two technologies for bioethanol obtaining from wood waste by use of two different types of hydrolysis: acid hydrolysis and enzymatic hydrolysis. The research, involved the study of various process parameters, the development of analysis methods for the fractions resulted in every stage, the optimization of the overall process for the determination of optimum parameters in order to improve each stage of the conversion to bioethanol.

The technologies for conversion of wood waste to bioethanol consist in three stages: pretreatment, hydrolysis and fermentation.

The thesis presents a modern method for pretreatment of wood waste: pretreatment with hot water (autohydrolysis). This pretreatment method uses only water at high temperature and pressure, without any chemicals for the separation of the wood waste in components. The chemical and enzymatic hydrolysis of cellulose were studied and compared, both in presence and absence of lignin in order to evaluate its influence on the process.

The thesis deals mainly with the development and validation of new analytical methods for analysis and quantification of carbohydrates and secondary compounds resulted in every stage of the wood treatment.

The thesis consists in three parts: the theoretical (Chapters 1-2) presents a literature review of the existing methods for obtaining bioethanol from different types of biomass, and a brief overview of pretreatment methods, hydrolysis and fermentation; the second part (Chapter 3-7) presents the original contribution of the thesis and the third part (Chapter 8) presents the experimental methods.

ORIGINAL CONTRIBUTIONS

CHAPTER 3 PRETREATMENT OF WOOD WASTE

3.1 The principle of water pretreatment technique

In accordance with the objectives of the thesis, the wood pretreatment by autohydrolysis was studied for separation of silver fir wood components, specific wood species for our region. Autohydrolysis was studied at laboratory level using a pressure reactor tested at different temperatures and residence time. For these experiments, silver fir wood was used as raw material.

In the last period of time, an increasing focus on green chemistry methods was noticed. The pretreatment method used in this study is a green method because the extraction of hemicelluloses was carried out with hot water without the use of chemical reagents. The carbohydrates from hemicelluloses are dissolved as soluble oligosaccharides and can be separated from insoluble fraction of cellulose and lignin.

3.2 Pretreatment of wood waste

The wood waste pretreatment stages are presented in Figure 3.4.

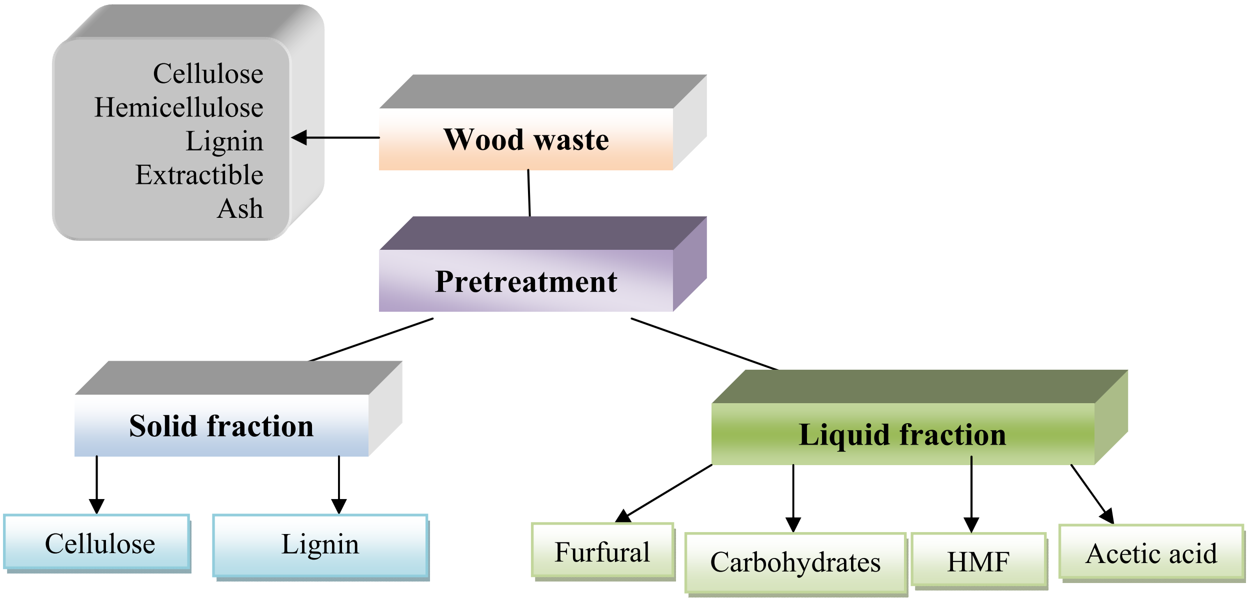


Figure 3.4 *Wood waste pretreatment stages*

The pretreatment of wood was carried out in a high pressure stainless steel reactor (Parr Instruments, Illinois, USA).

3.3 Characterization of cellulosic components resulted after pretreatment with water

After autohydrolysis pretreatment a solid fraction (cellulose and lignin) and a liquid fraction (monosaccharides, oligosaccharides, and degradation compounds) were formed. The composition of each phase was determined. New methods for carbohydrates, furfural and HMF analysis were developed and validated. The unhydrolysed oligosaccharides in liquid fraction were quantified as monosaccharides after hydrolysis.

3.3.1 Composition of raw material

The chemical composition of silver fir wood is presented in Table 3.2.

Table 3.2 Chemical composition of silver fir wood (expressed as mean values of triplicate analysis ± standard deviation) comparatively with the composition of other wood species reported in literature.

Wood species	Holocellulose	Cellulose	Hemicellulose	Lignin	Extractible	Ash
Present study	69.9	46.0 ± 0.7	23.9 ± 0.6	28.4 ± 0.3	1.3 ± 0.1	0.3 ± 0.1
Douglas-fir	69.0	42.0	27.0	28.3	2.5	0.2
Eucalyptus globulus	66.2	44.4	21.8	27.8	2.4	0.2
Pinus pinaster	58.1	39.2	18.9	28.5	2.8	0.2
Albies balsamea	69.0	42.0	27.00	29.0	1.8	0.2
Picea abies	66.8	41.7	25.1	28.3	1.6	0.2
Tsuga canadensis	64.0	41.0	23.0	33.0	2.8	0.2
Quercus ilex	71.2	43.0	28.3	16.3	n.d	n.d
Brutia pine	75.5	47.0	28.5	26.1	2.8	0.4
Olive wood	65.8	41.5	24.3	15.6	17.0	1.4

The analysis of the main constituents in the raw material showed that the silver fir wood samples contain about 70% carbohydrates (table 3.2). The content of moisture was 10%

and the content of total solids was 90%. The high carbohydrate content suggests that fir wood is a potential feedstock for bioethanol production.

3.3.3 Composition of solid fraction

After each experiment, the cellulose, lignin and hemicellulose contents in the solid fraction were determined (table 3.4).

Table 3.4 Solid yield and composition of solid fraction resulting after every stage, at different temperatures and residence time

Autohydrolysis condition	**180°C**			**190°C**			**200°C**		
	5	**10**	**15**	**5**	**10**	**15**	**5**	**10**	**15**
Solid yield (g/100 g raw material, on dry basis)	81.5	79.5	76.5	75.3	74.6	73.6	73.1	72.2	71.9
Solids compositions (g/100 g autohydrolysed wood, on dry basis)	93.4	96.9	89.4	92.9	98.9	96.9	95.6	98.2	94.4
Cellulose	48.6	56.6	50.6	52.3	57.8	55.9	56.3	59.5	54.9
Hemicellulose	6.2	4.7	4.2	3.8	3.3	2.5	1.1	0.0	0.0
Lignin	38.6	35.6	36.6	36.8	37.8	38.5	38.2	38.7	39.5

The results show that solid yield (71.9–81.5%) of pretreated wood decreases with the increase of pretreatment temperature, due to the hydrolysis of hemicellulose in the liquid fraction.

3.3.4 Composition of liquid fraction

Hemicellulosic fraction was analysed for content of monosugars, furfural, 5-hydroxymethylfurfural, oligomers sugar and acetic acid.

For the analysis of carbohydrates, furfural and HMF new chromatographic analytical methods were developed and validated. The instrumentation and validation report of each analytical method (limit of detection, limit of quantification, accuracy, robustness, linearity), were presented.

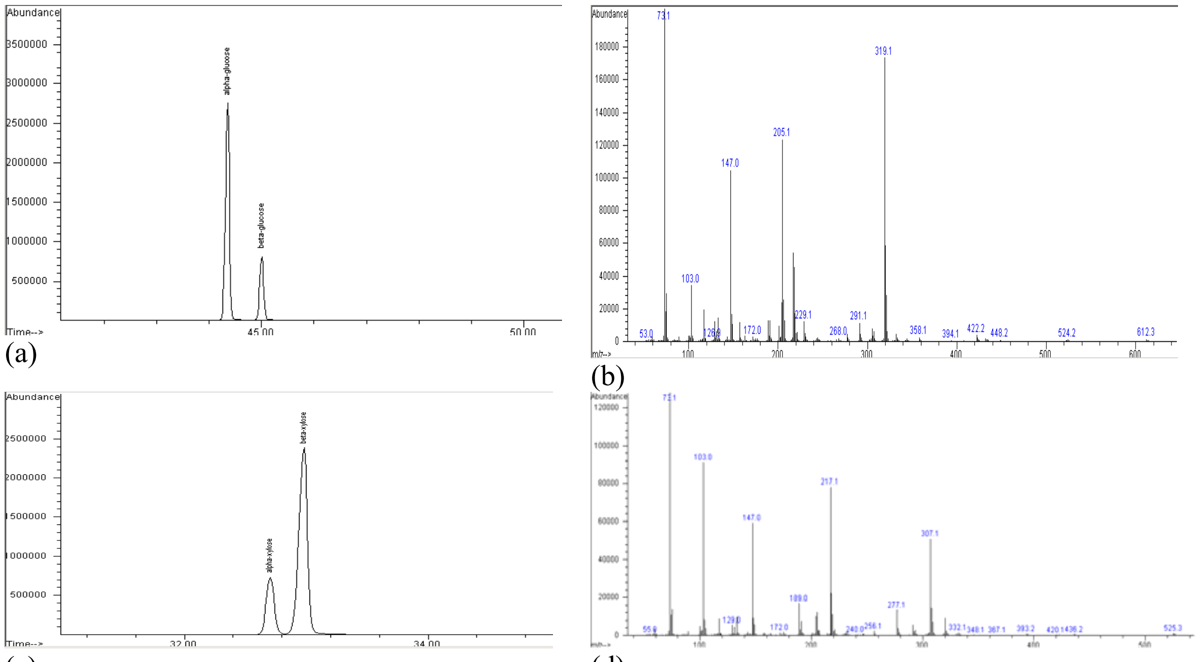
3.3.4.1 Identification and quantification of carbohydrates from hemicellulose by GC-MS

The aim of this study was development and validation of a new method for carbohydrates analyses obtained from woody biomass, as a step in the bioethanol production. The carbohydrates in wood were quantified by gas chromatography (GC) after derivatization. The samples were prepared by liquid-liquid extraction, derivatized by oximation and silylation and analysed by gas chromatography-mass spectrometry (GC-MS). Monosaccharide derivatives were identified by their GC retention time and MS fragmentation. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was used as derivatization reagent to prepare trimethylsilyl derivatives of hydrolysis products of hemicellulose after pretreatment. The sugar standards analysed in this study and their characteristics are given in Table 3.6.

Tabel 3.6 Characteristics of sugar standards analysed by GC-MS as oxime-TMS derivatives

Compounds	**Molecular formula**	**Molecular mass**	**Molecular mass – oxime-TMS**	**Retention time (min α-, β-)**	**m/z**
D-xylose	$C_5H_{10}O_5$	150	569	32.710, 32.985	73,103, 217
D-arabinose	$C_5H_{10}O_5$	150	569	33.002, 33.177	73,103, 217
D-galactose	$C_6H_{12}O_6$	180	627	43.853, 45.050	73,319, 205
D-mannose	$C_6H_{12}O_6$	180	627	44.026, 44.955	73,319, 205
D-glucose	$C_6H_{12}O_6$	180	627	44.363, 45.010	73, 319,205

Hexoses (glucose, galactose and mannose) contain five hydroxyl-groups, thus six-TMS derivatives are formed. Pentoses (arabinose and xylose) contain four hydroxyl-groups thus five-TMS derivatives are formed. Each sugar standard was analysed individually by oximation and silylation in order to determine the retention time of each isomers (figure 3.13).



(a) (b) (c) (d)

Figure 3.13 *GC-MS data from monosaccharide standards: (a) GC-MS chromatogram for a derivatizated solution of D-glucose; (b) mass spectrum of α-D-glucose-oxime-TMS; (c) GC-MS chromatogram for a derivatizated solution of D-xylose; (d) mass spectrum of α-D-xylose-oxime-TMS*

The GC chromatogram of the carbohydrates obtained after oximation and derivatization of the sugar mixture with the internal standard is shown in figure 3.14.

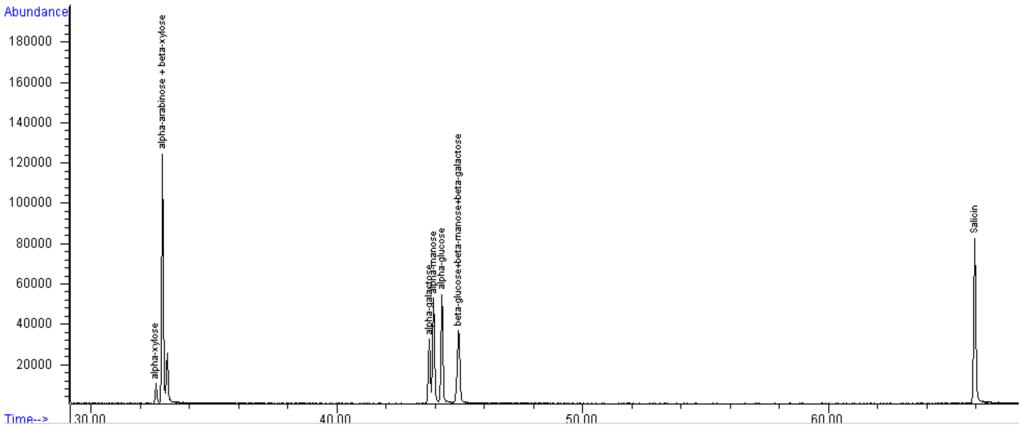


Figure 3.14 *The chromatogram of TMS derivatives of the reference compounds (150 µg ml^{-1}) determined by GC*

Due to very close retention times, the peaks of some compounds overlap and some isomers cannot be separated due to the epimerisation process. The peaks of *α*-arabinose and *β*-xylose cannot be separated and also *β*-glucose, *β*-mannose and *β*-galactose have the same retention time.

Analysis of carbohydrates from wood

The chromatogram of carbohydrates from hemicellulose obtained from wood by autohydrolysis pretreatment method is presented in figure 3.15. The carbohydrates were identified by coincidence of their retention time and mass spectra with those of pure standards.

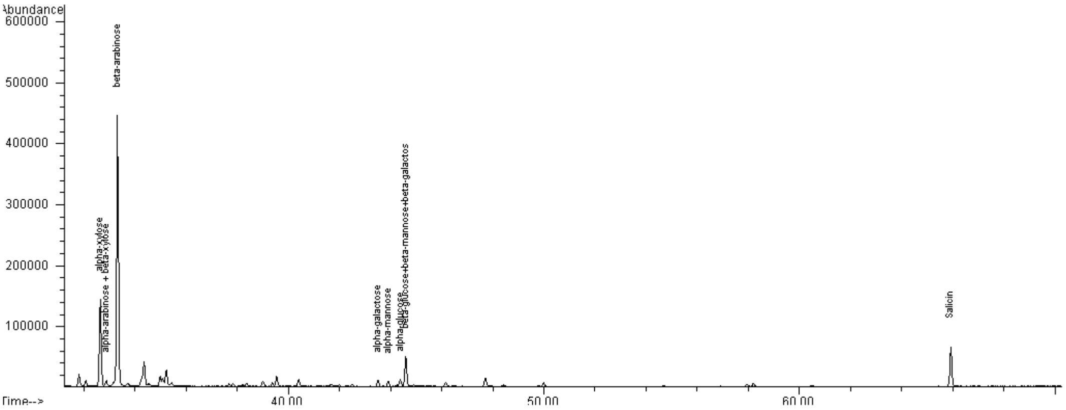


Figure 3.15 *The chromatogram of TMS derivatives of the hemicellulose obtained from wood (190°C, 10 min)*

Figure 3.17 (a) and (b) presents the hexose and pentose content in function of pretreatment conditions.

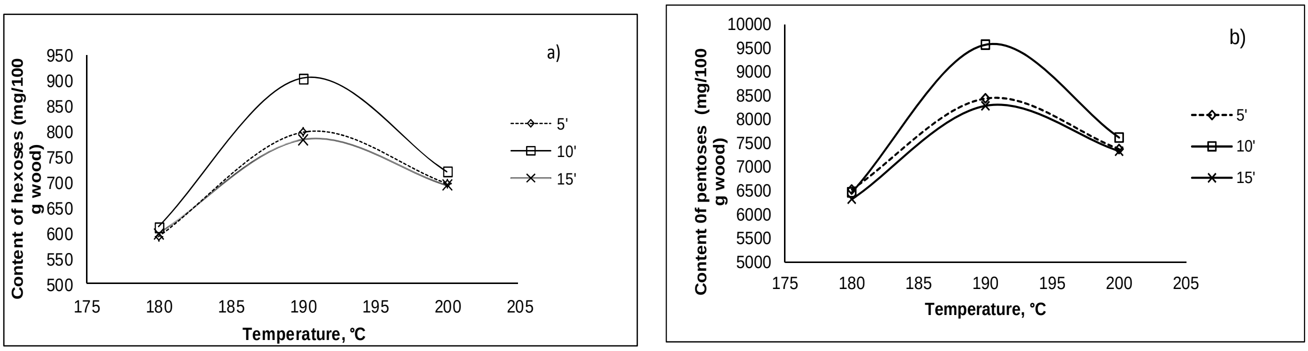


Figure 3.17 *Composition of hexoses and pentoses in filtrates in different pretreatment conditions*

Figure 3.19 presents the contents of sugars from the hemicellulosic fraction and sugars recovery depending on the pretreatment conditions. Hemicellulose is recovered as sugars (monomers and oligomers) are 34–47%.

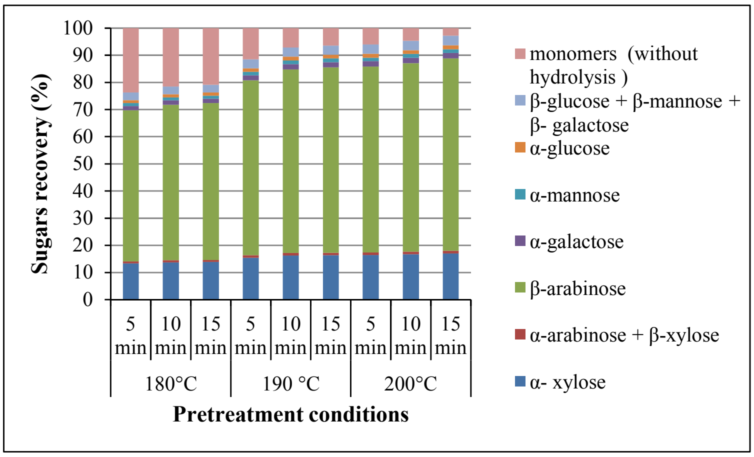


Figure 3.19 *Content of sugar monomers and oligomers from the hemicellulosic fraction*

3.3.4.2 Analysis of 5-hydroxymethylfurfural by GC-MS

The aim of this study was to develop and validate a new gas chromatography – mass spectrometry (GC-MS) method used for the analysis of 5-hydroxymethylfurfural (HMF) in water soluble wood fraction, obtained after autohydrolysis pretreatment. Two different methods were used for HMF extraction from water soluble wood, before gas chromatographic analysis: liquid-liquid extraction (LLE) with dichloromethane and solid phase extraction (SPE) method.

The analytical parameters of the two methods were compared. HMF is obtained from the degradation of hexoses (glucose, mannose and galactose) present in wood.

Figure 3.21 shows the chromatogram of 5-hydroxymethylfurfural standard solution. The retention time of HMF was 9.322 min.

The derivatization procedure and the extraction method were optimized for each method. HMF contains one hydroxyl-group, thus one-TMSi derivate is formed. The mono derivate for HMF was evidenced by the presence of *m/z* 183, 169, 109, indicating silylation of hydroxyl-group. The *m/z* 183 corresponding to the ion $[M-CH_3]^+$ was selected for quantification and molecular ion at 169 corresponding to $[M-COH]^+$ and 109 to the $[M-OTMS]^+$.

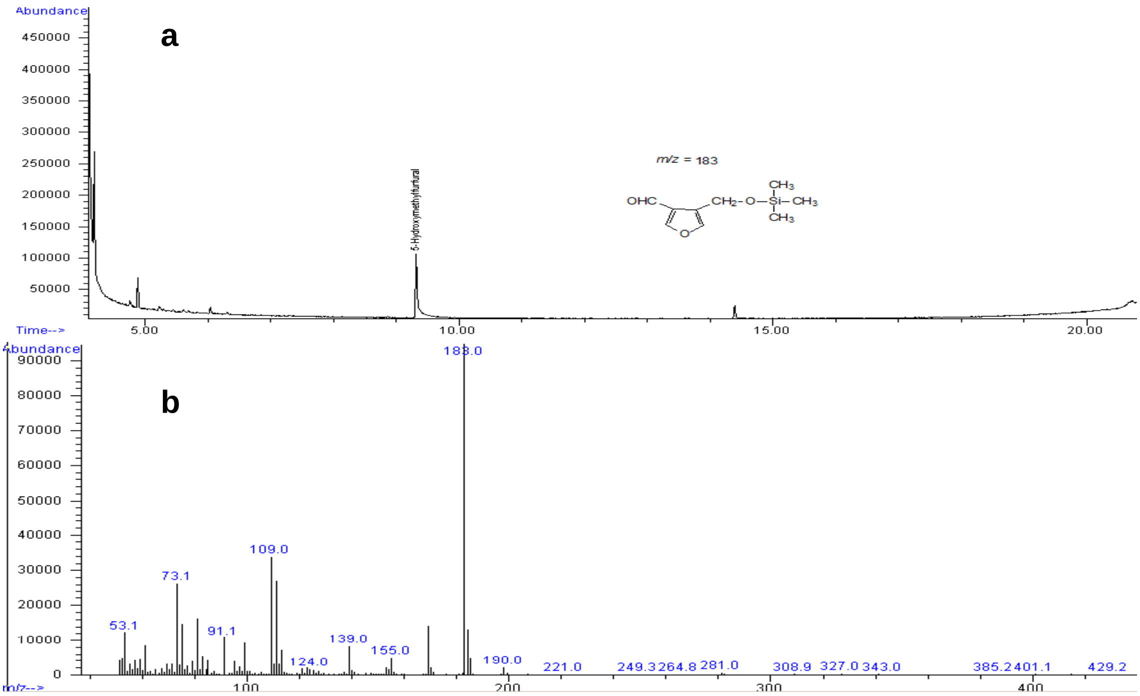
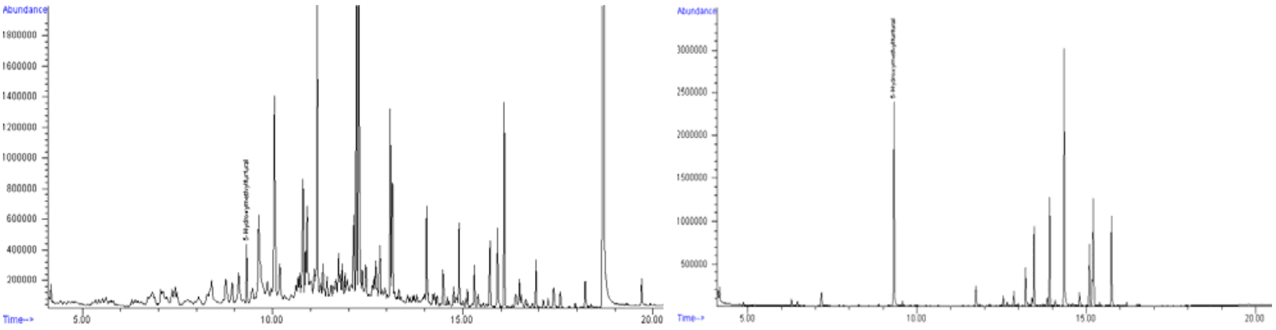


__Figure 3.21__ GC-MS data for HMF standard solution: (a) GC-MS chromatogram for a derivatizated solution of HMF; (b) mass spectrum of TMSi-HMF

Comparison of SPE and LLE for extraction of HMF from wood – comparative study

After each extraction procedure the HMF content of each sample was determined. The GC chromatogram of HMF obtained after derivatization of the liquid fraction resulted after autohydrolysis pretreatment (190°C) using LLE and SPE extraction is shown in figures 3.22 a and b.



a) b)

__Figure 3.22__ The chromatogram of the TMSi-HMF compounds obtained by: (a) LLE and (b) SPE extraction method

The content of HMF obtained in hemicellulosic fraction in function of the pretreatment conditions is shown in Figure 3.23.

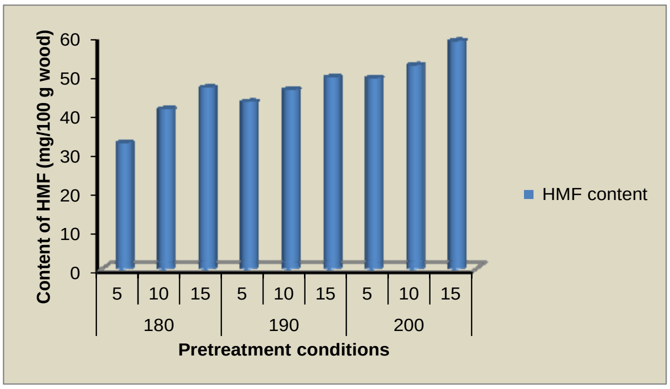


***Figure 3.23** Composition of HMF in hemicellulosic fraction in function of the pretreatment conditions*

3.3.4.3 Analysis of furfural by GC-MS

Two extraction methods based on liquid-liquid (LLE) and headspace solid phase microextraction (HS-SPME) were evaluated for the analysis of furfural in the hemicellulose hydrolysate. For each method, the linearity, detection limits, quantification limits, recovery and reproducibility were determined (table 3.13).

Table 3.13 Linearity range, correlation coefficient, detection limits, quantification limits and recovery obtained for each method for the analysis of furfural from hemicellulose hydrolysates

Method	**Linearity**		**Detection limit ($\mu g\ ml^{-1}$)**	**Quantification limit ($\mu g\ ml^{-1}$)**	**RSD (%) n=5[a]**	**Recovery (%) (mean±SD, n=5)**
	Range ($\mu g\ ml^{-1}$)	**R^2**				
LLE	10 –350	0.995	3	9	10.5	78 ± 6.3
HS-SPME	0.1 - 35	0.999	0.03	0.09	7.3	95 ± 5.9

[a] Relative standard deviation

Content of furfural in the hemicellulosic hydrolysates

Furfural was determined by GC-MS after liquid-liquid extraction and headspace SPME, methods without derivatization.

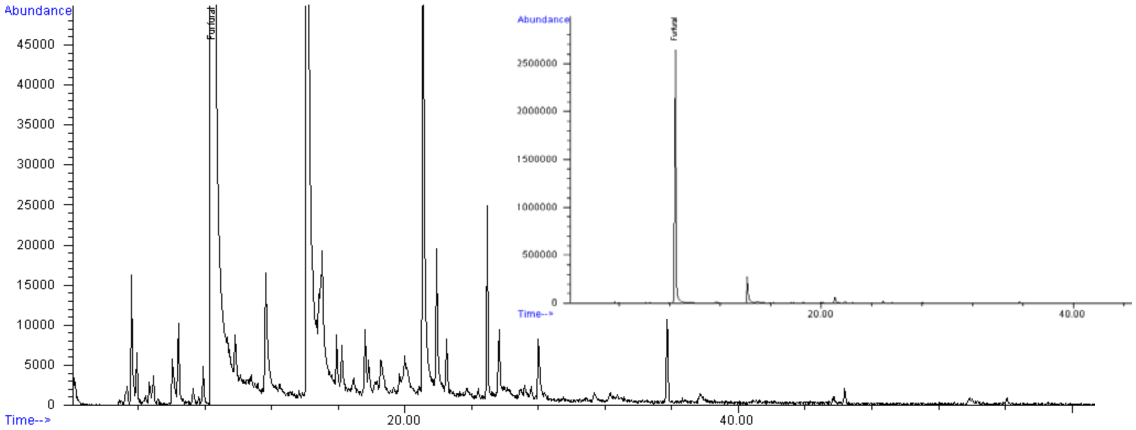


Figure 3.26 *Chromatogram of furfural from hemicellulosic fraction obtained after HS-SPME extraction of samples autohydrolysed at 190°C for 10 min*

Figure 3.27 shows the furfural contents in hemicellulose fraction, in function of the reaction conditions.

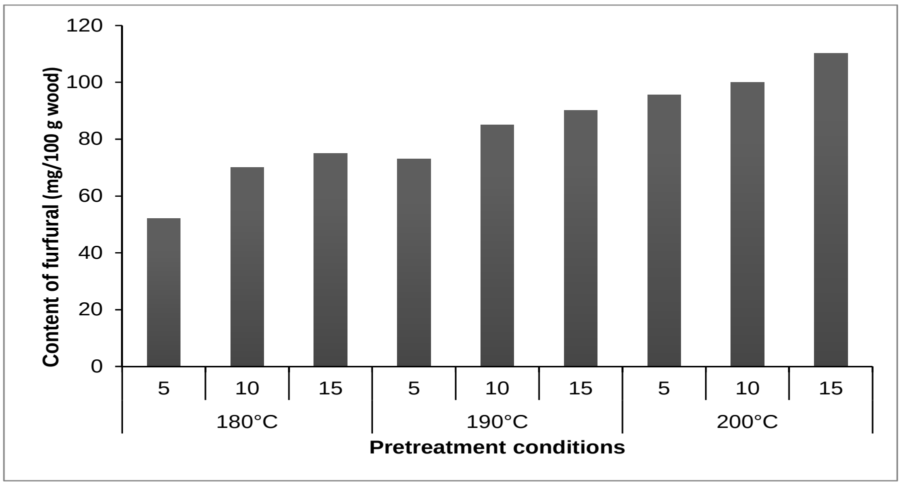


Figure 3.27 *Furfural contents of hemicellulosic fraction*

Furfural contents of hemicellulosic fraction obtained from wood increase with temperature and reaction time.

3.3.6 Statistical analysis by adaptive neural fuzzy interference (ANFIS)

The variation of components in the hemicellulosic and cellulosic fraction (dependent variables) and the independent variables were established by using a fuzzy neural model based on equation (3.18) [181]:

$$Y_e = \frac{\sum_{l=1}^{m} y^l R_l}{\sum_{l=1}^{m} R_l} \qquad (3.18)$$

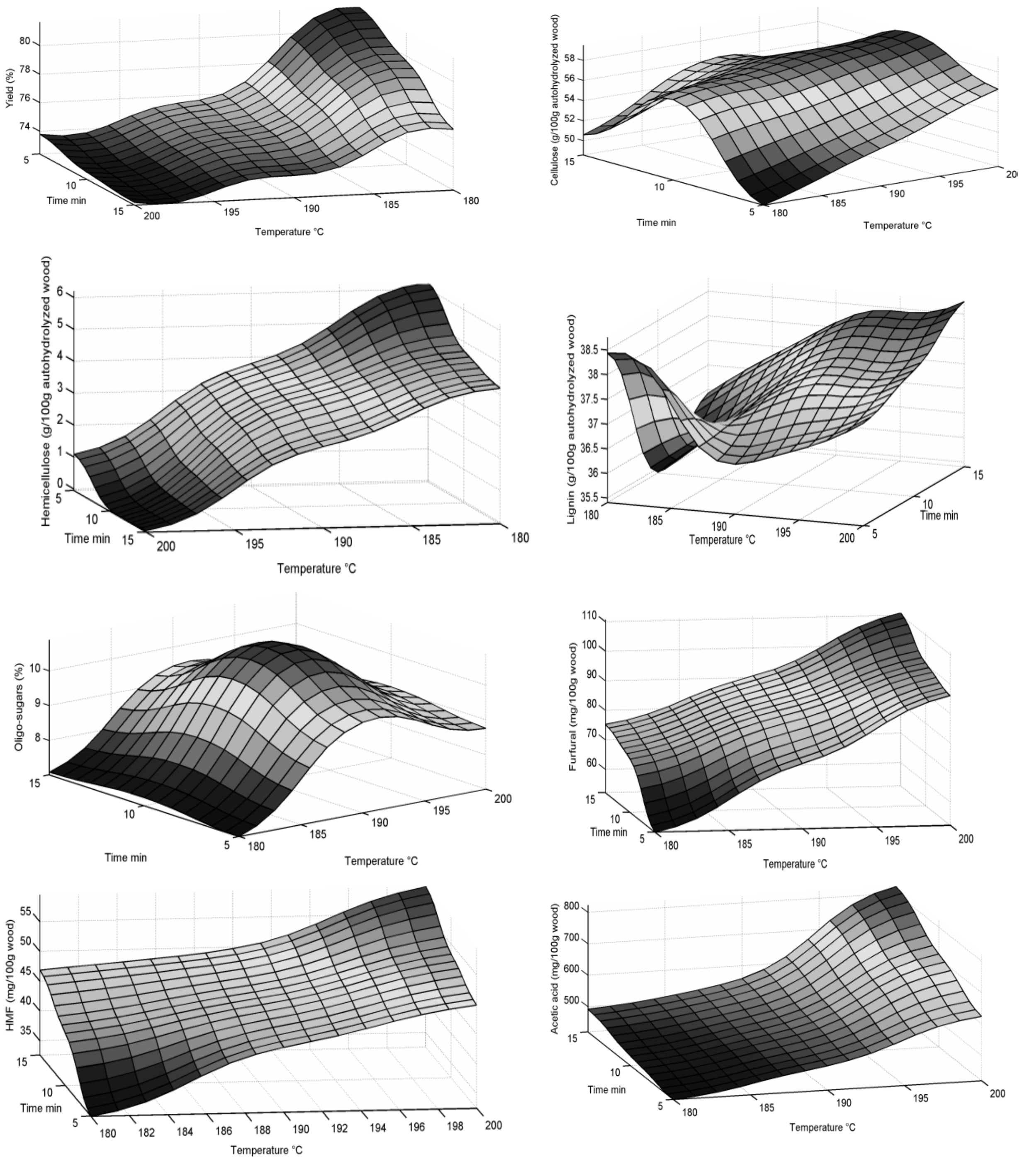
where: Y_e is the estimate value of output variable, m the number of rules, n the number of input variables, y^l the defuzzifier, R_l fuzzy rule (is defined by the product of n membership functions).

With two independent variables, one can establish nine fuzzy rules (R_i) with a Gaussian membership functions for independent variables with three levels (low, medium and high) of the temperature and time. The three levels are for one independent variable.

The experimental data obtained by analysis of solid fraction resulted after autohydrolysis pretreatments were processed using the ANFIS model. The proposed model used two independent variables (temperature and time) and one dependent variable processed with Gaussian membership functions at three levels (low, medium, and high) for estimation the parameters.

In Figures 3.29 -3.36 the variations of parameters depending on temperature and time is presented.

The composition of solid and liquid fraction were modelled as a function of the independent variables (temperature and time) using the ANFIS Edit tool in Matlab 7.0. Software ANFIS Edit Matlab 7.0 was used to develop a fuzzy neural model that reproduces the experimental results of the dependent variables with errors below 1%.



Figures 3.29 -3.36 *Composition of liquid fraction and solid fraction resulted after pretreatment as a function of independent variable*

CHAPTER 4 DELIGNIFICATION OF PRETREATED WOOD WASTE WITH SODIUM CHLORITE

The aims of this chapter were obtaining cellulose from wood by using two methods: chlorite delignification after pretreatment and direct delignification of wood. Sodium chlorite delignification was used for lignin removal from non-pretreated wood. Direct delignification of dried wood with sodium-chlorite was made for the extraction of holocellulose (mixture of cellulose and hemicellulose).

In this chapter the autohydrolysis pretreatment for hemicellulose removing and the delignification with sodium-chlorite for lignin removement in order to obtain pure cellulose that is enzymatically hydrolysed to sugars was studied. Pretreatment with sodium chlorite degrade the lignin by generation of chlorine dioxine (ClO_2), an oxidation product of chlorous acid ($HClO_2$) and hypochlorous acids (HOCl) produced during pretreatment. Pretreatment with sodium chlorite was a selective method for lignin and cellulose degradation if the reaction medium is neutral. In these conditions, acetic acid was used for an acid reaction medium for preventing degradation of cellulose. Solid yield after autohydrolysis method and solids yield after delignification methods are shown in Table 4.1.

Table 4.1 Composition of solid fraction obtained after autohydrolysis pretreatment and delignification of pretreated wood (a) and holocellulose (b)

Autohydrolysis condition	**180°C-a**	**190°C-a**	**200°C-a**	**No pretreated, delignified -b**
Solid yield (g/100 g raw material, on dry basis)	79.5	74.6	72.2	-
Solids compositions (g/100 g autohydrolysed wood, on dry basis)	96.9	98.8	98.2	-
Cellulose	56.6	57.8	59.5	
Hemicellulose	4.7	3.3	0.0	
Lignin	35.6	37.8	38.7	
Solid yield (g pretreated and delignified wood/100 g raw material, on dry basis)	47.6	42.9	39.5	76.1
Solids compositions (g/100 g pretreated and delignified wood)	96.8	98.8	96.3	92.8
Cellulose	85.0	90.1	93.0	59.9
Hemicellulose	4.4	2.5	0.0	26.7
Lignin	7.3	6.0	3.2	6.0

The results show that solid yield (79.5–72.2/ 100 g raw material) of pretreated wood decreases with the increase of pretreatment temperature, due to the hydrolysis of hemicellulose in the liquid fraction. The content of cellulose (56.6–59.6%) and lignin (35.6–38.7%) in the autohydrolysed wood confirmed that both components were recovered in solid fraction. The delignification yield decreases with the increasing of pretreatment temperature due to the solubilisation of lignin. In Figure 4.2 the composition of solids resulted after every process is shown.

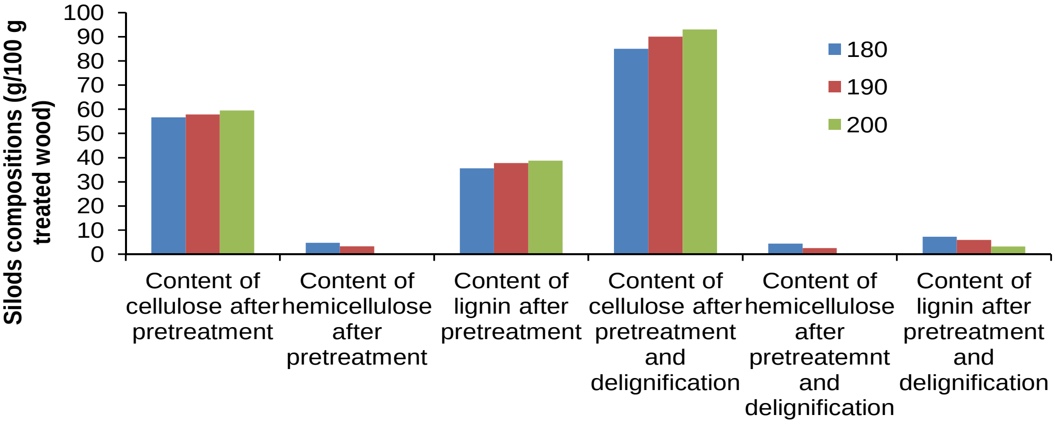


Figure 4.2 *The influence of temperature on solids compositions resulted after every treatment applied*

The results of delignification yield varied in the range 39.5–47.6 g pretreated and delignified wood/100 g raw material for the process with pretreatment by comparison with the process without pretreatment where, the delignification yield was 76.1 g wood delignified/100 g raw material; this can be explained by the presence of cellulose and hemicellulose in the direct delignification process while, in the pretreated and delignified process, only cellulose and small amount of hemicellulose and lignin were obtained.

CHAPTER 5 ACID HYDROLYSIS OF PRETREATED WOOD WASTE

Autohydrolysis involve separation of cellulose in solid fraction at high temperature and high pressure. The pretreated material is separated by filtration in solid and liquid phase. Solid fraction (which contains cellulose and lignin) can be hydrolysed or delignified for lignin removal and then hydrolysed to glucose. The aims of this chapter was the glucose obtaining from pretreated wood and from pretreated and delignified wood by acid hydrolysis with sulphuric acid in two hydrolysis stage. Figure 5.1 shows the experimental procedures used for glucose obtaining.

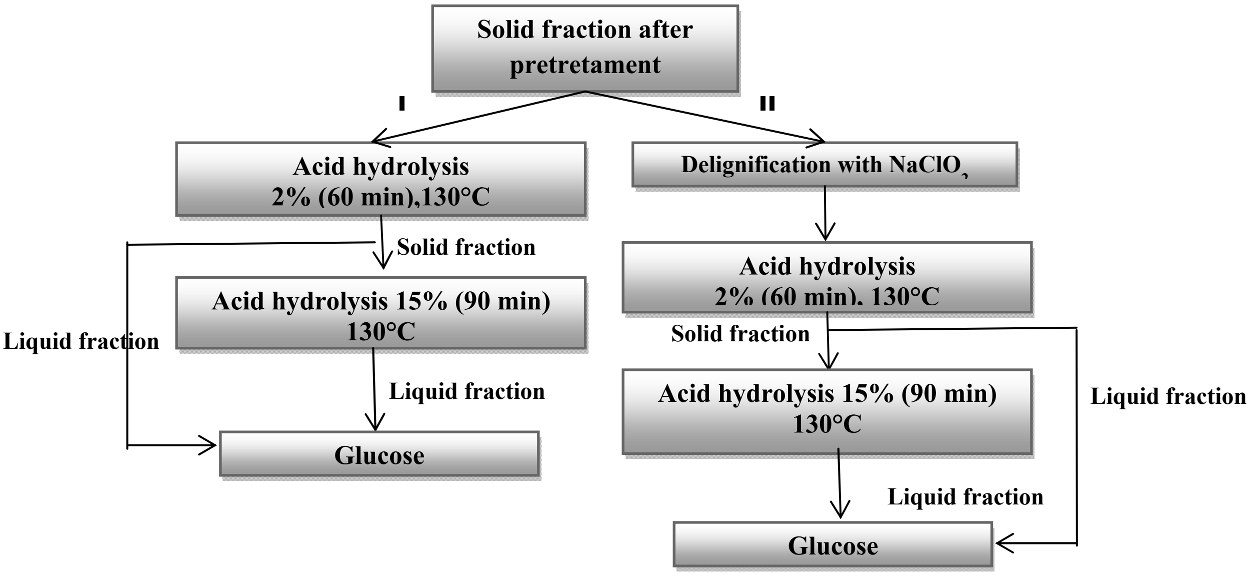


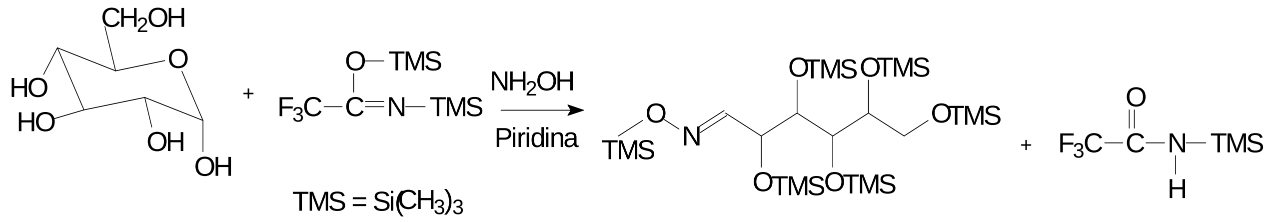
Figure 5.1 *Glucose obtaining from solid fraction resulted after pretreatment*

5.2 Analytical methods used for glucose determination in hydrolysate

The analysis of glucose from acid hydrolysate was made by two methods: **analysis of glucose by GC-MS** for identifying the presence of both α-and ß-glucose isomers (subchapter 5.2.1 - GC-MS methods for analysis of glucose from cellulosic fraction resulted after hydrolysis) and **analysis of reducing sugars** by spectrophotometric method (subchapter 5.2.2 - spectrophotometric method for reducing sugars determination from hydrolysate).

5.2.1 GC-MS methods for analysis of glucose from cellulosic fraction resulted after hydrolysis

The aim of this subchapter was developing and validation of a new method for glucose analysis. The method is based on derivatization of sugars with a silylation agent. As silylation agents HMDS (hexamethyldisilasan) in trifluoroacetic (TFA) and BSTFA were used. The glucose quantification was made by liquid-liquid extraction followed by GC-MS detection. The silylation of hexose by BSTFA is presented in figure 5.2.



Scheme 5.2 *Chemical structure of the silylated derivative of glucose (BSTFA)*

The glucose was obtained from the wood biomass both in case of the pretreatment by autohydrolysis (in the hemicellulosic fraction) and in case of hydrolysis [173]. The separation of cellulose from hemicellulose requires a strong pretreatment. The autohydrolysis in the presence of water is a physical-chemical method for cellulose separation. The solid fraction obtained after pretreatment was hydrolysed by chemical or enzymatic methods.

Figure 5.2 (a) shows the chromatogram of glucose obtained after oximation with hydroxylamine hydrochloride in pyridine and BSTFA obtained by GC-MS.

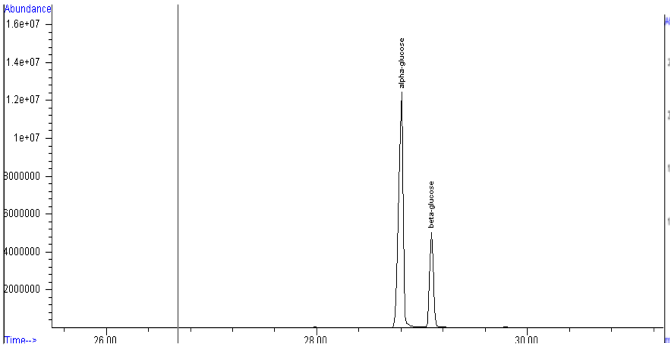


Figure 5.2 (a) *The SIM chromatogram of the glucose after oximation and silylation with BSTFA*

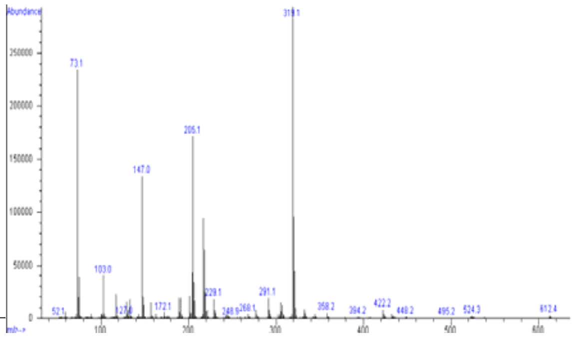
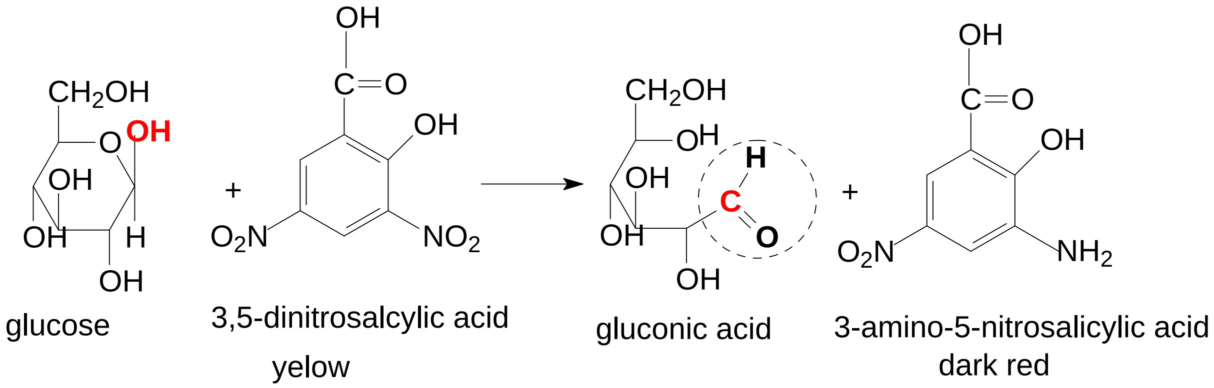


Figure 5.3 *The MS spectra of the ions for the quantitative and qualitative analysis of glucose silylation derivatives*

The limit of detection (LOD) for glucose was calculated as the concentration that corresponds to three standard deviations of the blank (3s criterion, 10 independent blanks for each analyte) [8]. The limit of detection was 0.222 µg for 1α-glucose and 0.171 µg for 1ß-glucose, respectively.

5.2.2 Spectrophotometric method for reducing sugars determination from hydrolysate

For the determination of carbohydrates a colorimetric method was used. The 3,5-Dinitrosalicylic acid (DNS) was used as colouring agent. The DNS solution is white, but in the presence of hexoses is transformed in 3-amino-5-nitrosalicilic of red colour.



Scheme 5.4 *Reaction of glucose with DNS*

Determination of DNS reducing sugars from hydrolysate by spectrophotometric method has the following advantages: is a direct reaction; measures the reducing capacity of glucose; uses a reaction which is based on colour change from yellow to dark red, with maximum absorbance at 540 nm; is a mole to mole reaction, the amount of reducing sugars are proportional with the amount of glucose.

The DNS method can determine the concentration of all reducing sugars from hydrolysis media, not only the glucose concentration.

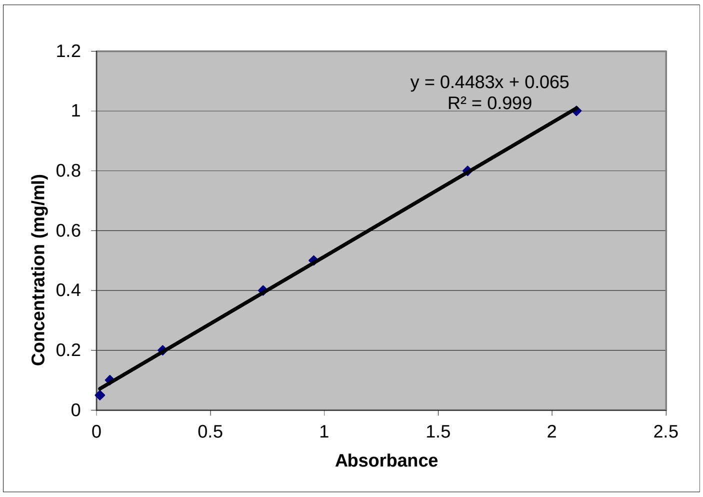


Figure 5.4 *Calibration curve for the determination of reducing sugars by DNS methods*

5.3 Acid hydrolysis of cellulosic components obtained from wood waste

The acid hydrolysis of cellulosic components obtained from wood was carried out using as raw material the cellulosic material resulted after autohydrolysis pretreatment at different temperatures and residence time and the pretreated and delignified wood. The acid hydrolysis experiments was carried out using the material pretreated by autohydrolysis at 180, 190 and 200°C and 10 minutes residence time in all cases (the optimal pretreatment conditions). Two-step acid hydrolysis method was used for acid hydrolysis of celluloses obtained from silver fir wood.

5.3.2 Sulphuric acid hydrolysis of pretreated wood

In the literature no data was found regarding the application of acid hydrolysis method on pretreated and delignified wood with sodium chlorite. In this chapter, the hydrolysis with dilute sulphuric acid in two-stage acid impregnation was used. Given the above considerations, acid hydrolysis reaction was done in two stages. In the first stage, 2% concentration of acid was used, followed in the second stage where the concentration of acid was increased to 15%. The hydrolysis conditions were selected to achieve a lower cellulose degradation rate.

Figures 5.5 b presents the acid hydrolysis yields determined from reducing sugars content for each stage of acid hydrolysis.

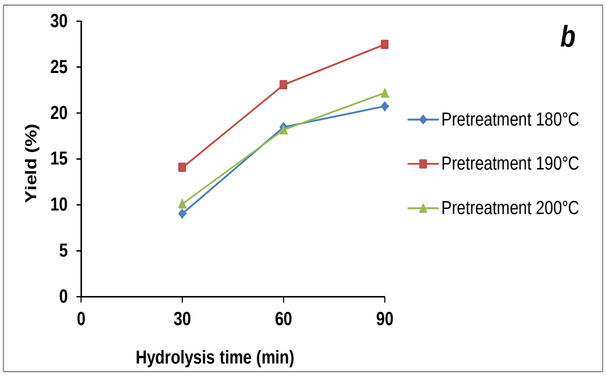


Figure 5.5 *Acid hydrolysis yields expressed as reducing sugar concentration obtained for: (b) hydrolysis with 15% sulphuric acid at 30, 60 and 90 min*

Figure 5.6 shows the chromatogram of hydrolysate obtained after hydrolysis in two-step acid hydrolysis.

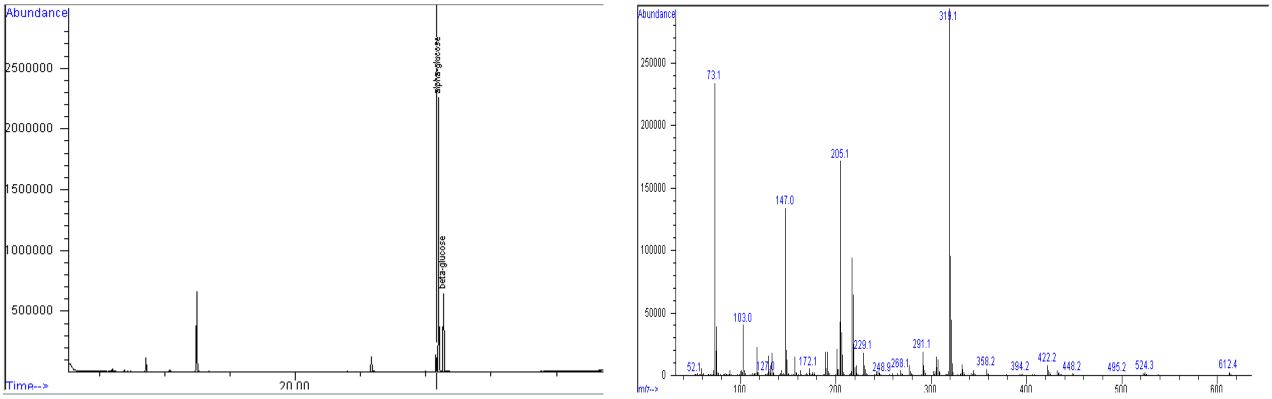


Figure 5.6 *GC-MS chromatogram of the derivatized solution of hydrolysates obtained after acid hydrolysis*

The two isomers were found in the hydrolysate in a 4:1 (α-glucose/ß-glucose) molar ratio. Acid hydrolysis of wood resulted after autohydrolysis pretreatment was done by two-stage hydrolysis. After the first hydrolysis stage (hydrolysis with 2% sulphuric acid) very low yield was obtained. In the second stage (hydrolysis with 15% sulphuric acid) the highest yield, 27%, was obtained at 190°C, which means that the presence of lignin in the acid hydrolysis acts as an inhibitor of hydrolysis.

5.3.3 Dilute sulphuric acid hydrolysis of pretreated-delignified wood

Figure 5.9 (a, b) shows the acid hydrolysis yields determined from the reducing sugar contents obtained by hydrolysis of pretreated and delignified wood.

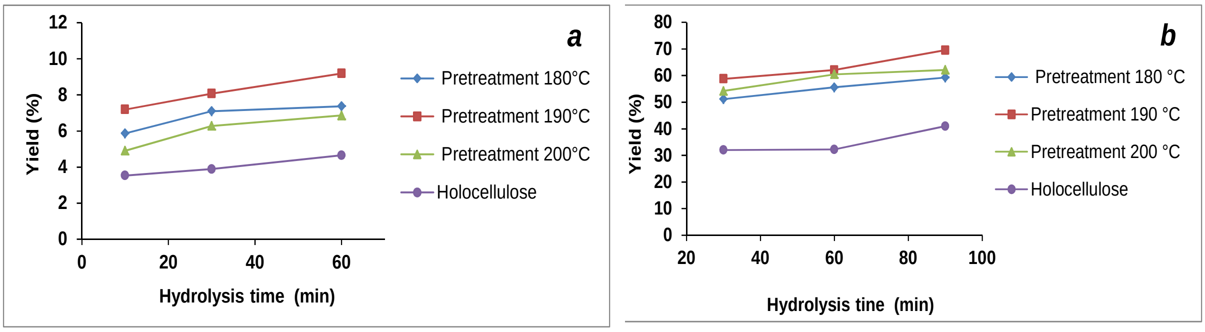


Figure 5.9 *Acid hydrolysis yields expressed as reducing sugar concentration obtained for: (a) hydrolysis with 2% sulphuric acid at 10, 30 and 60 min, and (b) hydrolysis with 15% sulphuric acid at 30, 60 and 90 min*

The acid hydrolysis yields were evaluated for 10, 30 and 60 minutes for hydrolysis with 2% sulphuric acid and for 30, 60 and 90 minutes for hydrolysis with 15% sulphuric acid. The low yield of acid hydrolysis in case of pretreated and delignified wood suggests that the low concentration of acid has reduced influence on cellulose; therefore it is necessary to increase sulphuric acid concentration to 15%.

5.3.4 The global yield of acid hydrolysis

Figure 5.10 shows the global yield of acid hydrolysis.

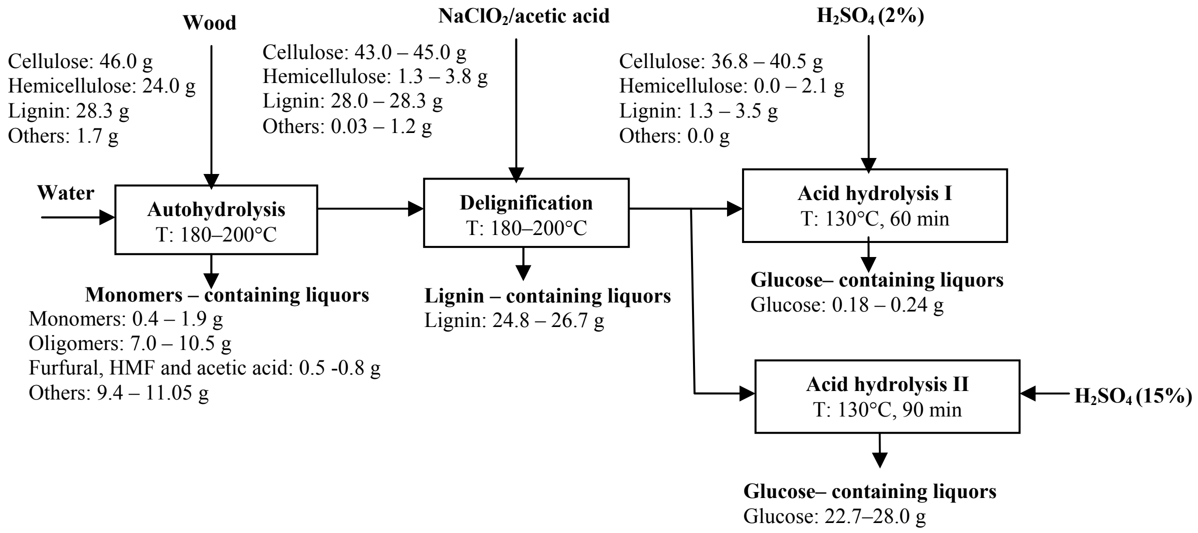


Figure 5.10 *Material balances for glucose obtaining by acid hydrolysis of pretreated and delignified wood in two-step hydrolysis method*

CHAPTER 6 ENZYMATIC HYDROLYSIS OF PRETREATED WOOD WASTE

Compared to acid hydrolysis, enzymatic hydrolysis is more promising because it not requires chemicals. Cellulase, the enzyme used for hydrolysis of cellulose complex, contains exoglucanase, endoglucanase, and beta-glucosidase.

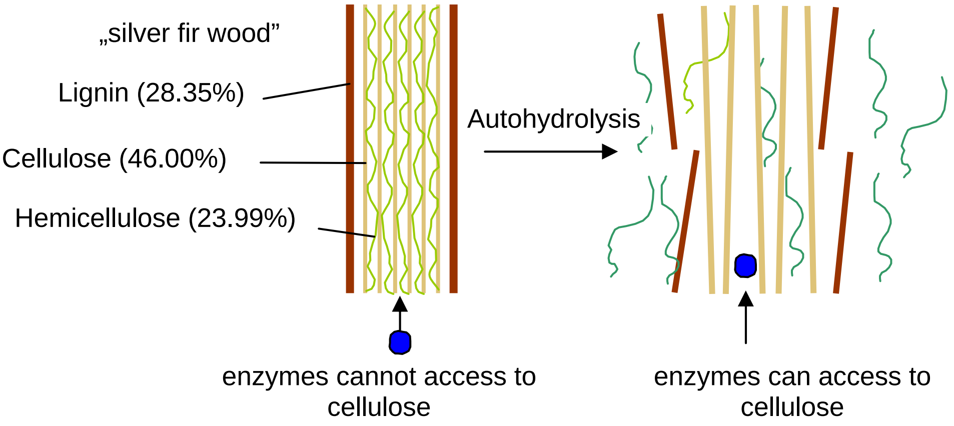


Figure 6.2 *Schematic representation of the wood structure before and after autohydrolysis pretreatment and enzyme access to cellulose*

The high content of lignin from wood is the main barrier for enzymatic hydrolysis of cellulose obtained from wood. The *Accellerase 1500* enzymes was used in all enzymatic hydrolysis experiments because its capability to hydrolyse cellulose and hemicellulose.

The aims of this chapter were obtaining cellulose by enzymatic hydrolysis of non-pretreated and autohydrolysis pretreated wood under different conditions and chlorite delignification for evaluation of lignin and hemicellulose in the enzymatic hydrolysis process.

6.2 Enzymatic hydrolysis of wood wastes

The pretreated samples were submitted to enzymatic hydrolysis varying the concentrations of substrate. In all experiments 1% and 2% (w/v) substrate solids were evaluated. The enzymatic hydrolysate was analysed by GC-MS for evaluation of components. The total concentration of sugars was determined as reducing sugars. The concentration of reducing sugars (expressed as glucose) resulted after 72 hours is shown in Table 6.1. The enzymatic hydrolysis yield was evaluated for 24, 48 and 72 hours.

Table 6.1. Concentration of reducing sugars (mg/g of substrate) obtained by enzymatic hydrolysis of pretreated wood

Substrate treatment	**Hydrolysis time (h)**					
	1% (w/v) concentration			**2% (w/v) concentration**		
	24	**48**	**72**	**24**	**48**	**72**
Pretreatment - 180°C	258.6	302.5	352.6	305.3	357.8	417.5
Pretreatment - 190°C	368.9	399.9	452.6	395.6	486.5	538.1
Pretreatment - 200°C	312.6	358.9	386.5	312.1	352.5	496.1

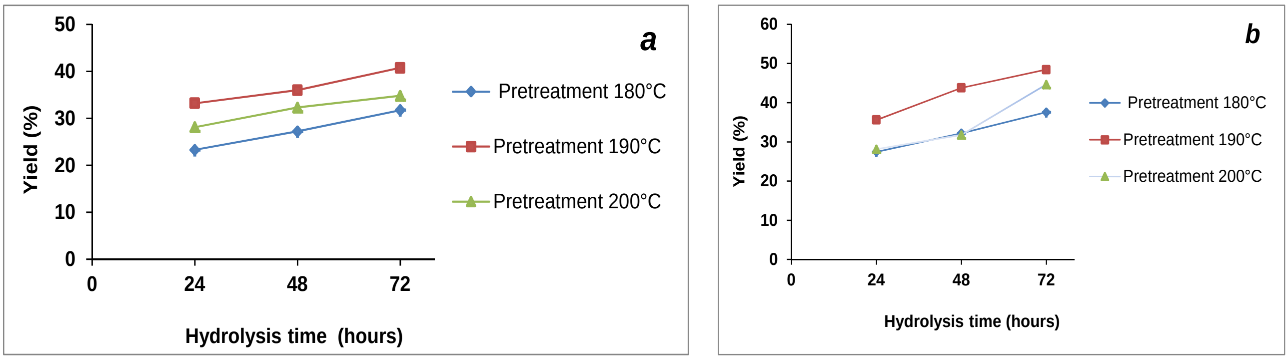


Figure 6.4 *Enzymatic hydrolysis yields at 24, 48 and 72 hours, expressed as ration of reducing sugar concentration obtained in the enzymatic hydrolysis and potential glucose in raw material, for: (a) 1% (w/v) substrate concentration and, (b) 2% (w/v) substrate concentration.*

6.2.2 Enzymatic hydrolysis of the pretreated and delignified wood

Non-pretreated wood and the cellulosic material resulted after pretreatment and delignification of wood was enzymatically hydrolysed to sugars. The concentration of reducing sugars (expressed as glucose) is presented in Table 6.2.

The results show that the composition of the wood significantly influences the enzymatic hydrolysis. A 40% yield was obtained for enzymatic hydrolysis with 1% (w/v) solid loading and 48% yield for enzymatic hydrolysis with 2% (w/v) solid loading. The highest enzymatic yield (91%) was obtained for silver fir wood after autohydrolysis at 190°C pressure (60 bar), 10 minutes residence time and 2% (w/v) solid loading, while a 80% yield was obtained for enzymatic hydrolysis with 1% (w/v) solid loading. The content of lignin in pretreated wood showed the limited accessibility of the enzyme to cellulose, suggesting that lignin is the restrictive component of enzymatic hydrolysis. The highest yield of the enzymatic hydrolysis in case of delignified wood suggested that the absence of lignin from delignified wood contribute to the easier access of enzymes to cellulose.

Table 6.2. Concentration of reducing sugars (mg/g of substrate) obtained by enzymatic hydrolysis of delignified silver fir wood and of autohydrolysis-delignified fir wood, at different temperatures, for 1% and 2% (w/v) concentration of substrate

	Concentration of reducing sugars (mg/g of substrate) for different substrates:					
	1% (w/v) concentration			**2% (w/v) concentration**		
Hydrolysis time (h) / **Substrate treatment**	**24**	**48**	**72**	**24**	**48**	**72**
Untreated	369.3	408.4	495.1	469.3	508.4	595.1
180°C	738.6	792.5	831.0	838.3	877.5	911.0
190°C	800.2	859.9	891.0	919.9	969.9	998.2
200°C	772.2	811.9	851.0	912.2	931.9	998

Figure 6.5(a) and 6.5(b) shows the enzymatic hydrolysis yields expressed as reducing sugar concentration after 24, 48 and 72 hours.

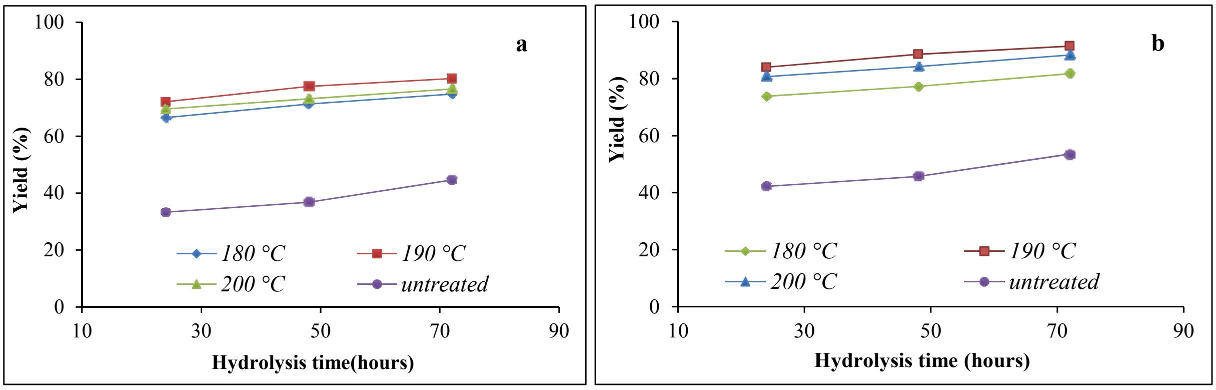


Figura 6.5 *Enzymatic hydrolysis yields at 24, 48 and 72 hours, expressed as ratio of reducing sugar concentration obtained in the enzymatic hydrolysis, and potential glucose in raw material, for: (a) 1% (w/v) substrate concentration and, (b) 2% (w/v) substrate concentration.*

The material balance for the wood pretreatment is shown in Figure 6.7.

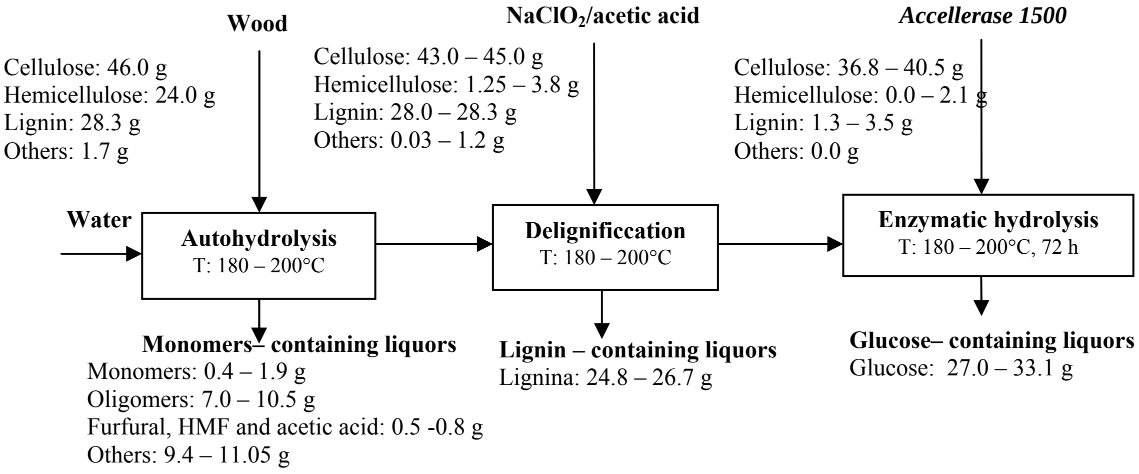


Figure 6.7 *Material balances for wood pretreatment*

The optimal conditions for sugar obtaining from silver fir wood in this work was defined by temperature (190°C), pressure (60 bar), residence time (10 minutes), solids loading (2%), and hydrolysis time (72 hours). These conditions led to obtaining of 33 g glucose/100 g wood. Overall, the processing of 100 g wood would result in the recovery of 74 g products. The sugars formed after enzymatic hydrolysis can be further fermented for bioethanol production.

CHAPTER 7 FERMENTATION OF SUGARS OBTAINED FROM WOOD WASTE

7.1 General principles of fermentation

In this chapter the production of ethanol by *Saccharomyces cerevisiae* assisted fermentation of sugars produced by acid and enzymatic hydrolysis of pretreated and pretreated-delignified wood was studied.

The obtaining of second generation bioethanol from fir wood includes the following steps:

- Autohydrolysis pretreatment (Chapter 3);
- Delignification of pretreated wood, lignin removal for easy accessibility of enzymes or acids to cellulose (Chapter 4);
- Acid hydrolysis (Chapter 5) of pretreated and pretreated-delignified wood or enzymatic hydrolysis of pretreated and pretreated-delignified wood (Chapter 6);
- Fermentation of glucose solution obtained both from acid hydrolysis and enzymatic hydrolysis (the results are presented in this chapter).

7.2 Fermentation of sugars obtained from wood to bioethanol

The ethanol determination by gas chromatography was validated. Ethanol was analysed by direct injection into the GC-MS through headspace. The concentration of ethanol was determined after 24, 48 and 72 hours for each hydrolysate subjected to yeast fermentation.

Figure 7.5 shows the fermentation yields determined after 24, 48 and 72 h.

Fermentation yields differ in case of acid or enzymatic hydrolysis of substrates. Fermentation of pretreated and acid hydrolysed wood (*experiment a*) gives a maximum yield of 32% (pretreatment temperature of 190°C) compared with the yield of fermentation for pretreated and enzymatic hydrolysed wood (*experiments c1* and *c2*) which giving a yield of

49% (solids loading of 1%), and 55% (solids loading of 2%). These results show that during the acid hydrolysis more inhibitors of hydrolysis are produced, due to the more drastic conditions (temperature and acid conditions for hydrolysis) compared to enzymatic hydrolysis conditions (neutral medium, low temperature and longer reaction time).

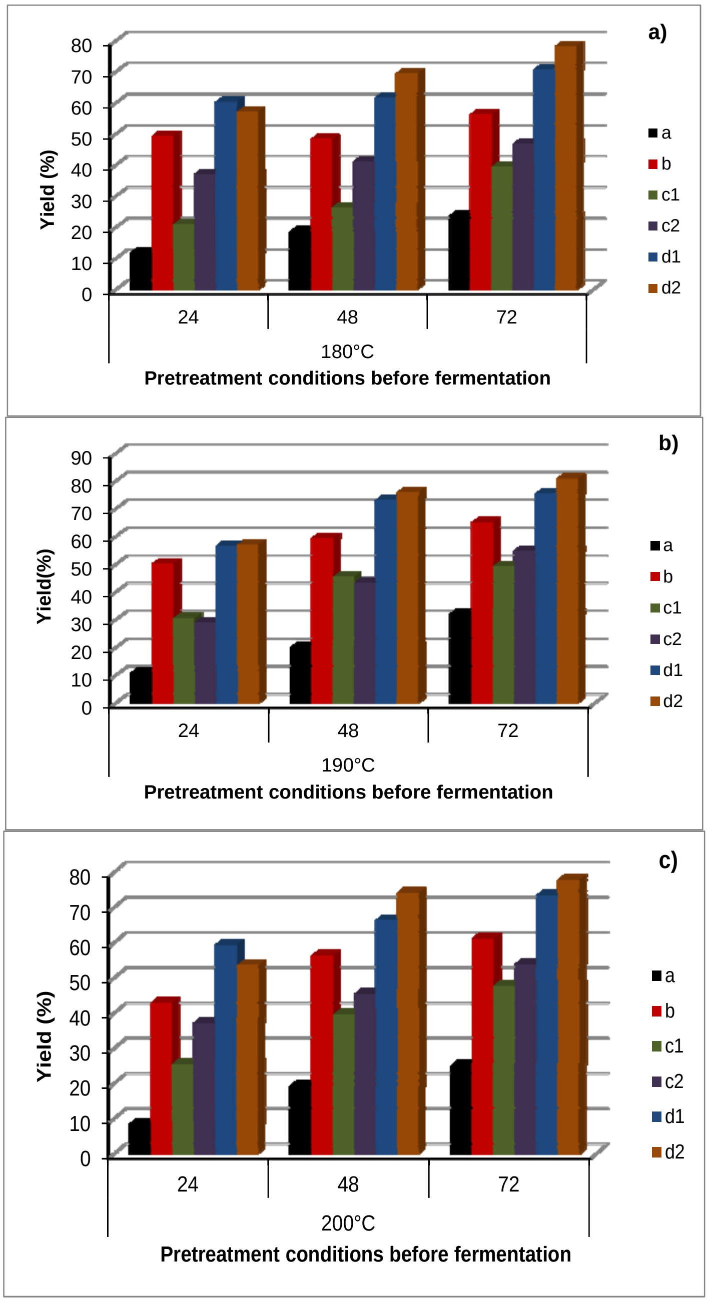


Figure 7.6 *Fermentation yield variation in function of treatment conditions before fermentation: (a) pretreatment temperature of 180°C, (b) pretreatment temperature of 190°C, (c) pretreatment temperature of 200°C (**a** - acid hydrolysis of pretreated wood, **b** - acid hydrolysis of pretreated and delignified wood, **c1** - enzymatic hydrolysis of pretreated wood (1%), **c2** - enzymatic hydrolysis of pretreated wood (2%), **d1** - enzymatic hydrolysis of pretreated and delignified wood (1%) and **d2** - enzymatic hydrolysis of pretreated and delignified wood (2%))*

The pretreated- delignified - enzymatically hydrolysed wood give higher fermentation yields 75% (*experiment d1*) and 80%, respectively (*experiment d2*) at the pretreatment temperature of 190°C, compared to the pretreated-delignified- acid hydrolysed wood which give a maximum fermentation yield of 65% (*experiment b*) at the pretreatment temperature of 190 °C. The pretreatment temperature has significant influence on delignification, enzymatic hydrolysis and fermentation. Generally, the higher fermentation yields were obtained at autohydrolysis treatment at 190 °C.

CONCLUSIONS

Two technologies for the production of bioethanol by superior valorisation of wood waste by chemical hydrolysis and enzymatic hydrolysis were developed. Due to its high content of cellulose and hemicellulose, as raw material the lignocellulosic biomass of silver fir wood was used.

For wood waste pretreatment a green method was developed. Wood hemicellulose extraction was achieved by autohydrolysis with at high temperatures and pressures. Using the autohydrolysis method, the components of wood were separate in two fractions: ***liquid fraction*** containing carbohydrates, soluble lignin and secondary products, and ***solid fraction*** containing cellulose and lignin.

The determination of cellulose, lignin and hemicellulose content in the recovered fraction after solid autohydrolysis, confirmed that lignin and cellulose was recovered almost quantitatively from the solid fraction.

Three new methods based on gas chromatography- mass spectrometry were developed and validated:

- identification and quantification of carbohydrates in hemicellulose fraction by GC-MS, using a double derivatization method
- analysis of 5- hydroxymethylfurfural
- analysis of furfural.

The performance parameters for each method: detection limits, quantification limits, working ranges, recovery studies of each method were established.

The analysis of carbohydrates composition from the hemicellulosic fraction showed that the mixture is composed from pentose and hexose.

Hydroxymethylfurfural (HMF) was analysed by two extraction methods: liquid-liquid extraction (LLE) and solid phase extraction (SPE). The two extraction methods were validated and the obtained results were compared. Compared with LLE method, SPE method has several advantages: superior recoveries, shorter time, low sample and reagent consumption thus is more environment-friendly. HMF content in wood ranged from 0.03 to 0.06 g/100 g wood, and its concentration increase with temperature and treatment time.

Furfural fraction from hemicellulose was analysed by two extraction methods: liquid-liquid and headspace SPME. Furfural content was within the range of 0.05 g – 0.1 g/100 g wood, depending on pretreatment conditions.

A mathematical model was developed for modelling the content of solid and liquid fractions resulted after pretreatment of wood as a function of the independent variables (temperature and time) using the ANFIS editing system of MATLAB 7.0 software. It was found that the mathematical model estimates the experimental results with an error lower than 1%.

Delignification of untreated and pretreated wood was carried out using sodium chlorite in acidic conditions in order to remove lignin before hydrolysis. Cellulose was obtained after the delignification. Yields of solid recovered after delignification with sodium chlorite ranged from 39.5 to 47.6 g treated and delignified wood/100 and raw material, depending on experimental conditions, which showed that after delignification it was obtained only cellulose. Effectiveness of the delignification methods was shown by determining the content of lignin removed (92-96%) after the delignification stage.

The cellulose obtained after delignification of pretreated wood was acid hydrolysed.

Two methods for glucose determination from wood hydrolysate were developed and validated:

- a new method for glucose analysis by gas chromatography;
- a spectrophotometric method for determination of reducing sugars.

There have been established the performance parameters of the methods: detection limits, quantification limits, working ranges, recovery studies. The method for glucose determination based on derivatization of glucose by oximation and silylation with BSTFA, showed the presence of both α-and ß-glucose isomers.

A method for glucose obtaining by two-step acid hydrolysis of pretreated wood was developed. The results showed that acid hydrolysis with 2% sulphuric acid concentration give low acid hydrolysis yield, while the acid hydrolysis with 15% sulphuric acid give higher yield

(27%), for wood pretreated at 190°C, leading to conclusion that the presence of lignin in the substrate acts as inhibitors of hydrolysis.

A method for glucose obtaining by acid hydrolysis of pretreated and delignified wood was developed. The highest acid hydrolysis yield (70%) was obtained for silver fir wood after autohydrolysis at 190°C, 60 bar pressure for 10 min residence time. The results showed that 15% concentration of acid is required in order to maximize the acid hydrolysis yield.

Methods for glucose obtaining by enzymatic hydrolysis of pretreated and pretreated-delignified wood were developed. The optimal conditions for glucose obtaining from silver fir wood in this work were: temperature (190°C), pressure (60 bar), residence time (10 minutes), solids loading (2%), and hydrolysis time (72 hours). These conditions led to obtaining of 33 g glucose/100 g wood. The highest enzymatic yield (91%) obtained for silver fir wood after autohydrolysis at 190°C with 2% (w/v) solid loading and lignin removal before enzymatic hydrolysis, showed that the treatment with sodium chlorite, besides lignin removing from substrate improves the accessibility of cellulosic material that becomes more susceptible to enzymatic action.

The technology for fir wood processing to bioethanol was carried out in the following steps:

- autohydrolysis pretreatment,
- delignification with sodium chloride of pretreated wood,
- acid or enzymatic hydrolysis,
- fermentation of acid or enzymatic hydrolysate to bioethanol with S. *cerevisiae*.

The ethanol was obtained by fermentation of acid or enzymatic hydrolysates with baker yeast *S. cerevisiae*. The fermentation of hydrolysate resulted after enzymatic hydrolysis of pretreated and delignified wood give the highest fermentation efficiency (81%) comparatively with fermentation of hydrolysate resulted after acid hydrolysis of pretreated and delignified wood (65%), for pretreatment temperature of 190°C, in both cases. The pretreatment temperature influence the delignification, hydrolysis and fermentation processes.

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