

INTEGRATED USE OF CARBOHYDRATES AND PHENOLIC STRUCTURES FOR THE FRACTIONING OF LIGNOCELLULOSIC RESIDUES

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Abstract

The paper presents the results of fractioning the biomass towards its bioconversion to ethanol for both – the carbohydrates resulting from hemicelluloses and from the ones of the lignocellulosic complex. Therefore not only methods of chemical hydrolysis were applied, but enzymatic ones too, using products developed in our own laboratories. Also, the possibility of turning into account the phenolic compounds – with high antioxidant potential – was closely looked into, as the phenols are the result of the enzymatic cleavage of the lignocellulosic edifice, rigidly structured. This treatment, unlike the acid hydrolysis, does not cause degradations in the aromatic structures, when submitted to high temperatures.

The distillation process, as well as the correction one for the ethanolic solutions that came both from the pretreatment phase and from the celluloso – lythical saccharification phase were performed in the same fashion, using the identical methodology. The results were, therefore, comparable and compared as considering the bioconversion yields and the „INVENTA AG” protocol, applied in Switzerland, through which a quantity of 95% ethylic acid is obtained, that is of 240 liters/tonne ligneous dried residue.

Keywords: lignocellulosic residues, ethylic alcohol, bioconversion

INTRODUCTION

Obtaining ethylic alcohol out of lignocellulosic residues in most advantageous conditions is a sensitive standard of all progress recorded in all areas of research, mainly throughout the last decades³.

This is due to the fact that the disassembly of the rigid macromolecular well consolidated of the biopolymers that are found throughout the biomass` composition, yet with a minimal degradation of the fermentable monomers was the beneficiary of unconventional technologies, such as the water

vapors explosion, gamma irradiation, microwave irradiation and was also subjected to high pressures 300-500 Mpa. On the other hand, when using genetic engineering, mutants were obtained, with a high bioconversion potential, capable of simultaneously fermenting, not only pentoses, but hexoses too, at temperatures of 60 - 70°C, thereby allowing the continuous elimination of alcohol as vapors, therefore substantially lowering the energetic costs in the distillation process and when correcting the alcohol obtained through bioconversion. Had been studied, at a micro pilot level the possibility of recovery the lignocellulosic residues in integrated phases of chemical pretreatment, enzymatic saccharification and oligophenol recovery as a result with sulfuric acid 4% pretreatments. The sugars resulted in the pretreatment phase were fermented with *Pachysolen tannophilus* yeast, strain CBS-4044 NRRLY-2460 (Genencor Co. – internal patent). The remaining lignocellulosic residue was treated with cellulases obtained from *Trichoderma viridae*, strain 3196 ICA (according to the Gh. Mencinicopschi technique, Institute of Food Chemistry (ICA) internal standard)^{3,4}.

MATERIALS AND METHODS

Materials

Biotransforming cellulosic materials using either commercial enzymatic products or microorganisms which produce cellulases leads to obtaining simple chemical compounds from which, through numerous fermentative processes (aerobic or non – aerobic) a wide variety of compounds might be obtained, with value of dietary supplement, of fodders, biofuels, solvents, enzymes etc. Considering these opportunities, the present study targets the analysis and the optimization of the biotechnological conditions of bioconverting carbohydrate polymers of lignocellulosic residues into ethanol.

Microorganisms

- ✓ *Pachysolen tannophilus* strain CBS-4044 NRRLY-2460
- ✓ *Trichoderma viridae*, strain 3196 Institute of Food Chemistry (ICA)
- ✓ *Trichoderma reesei* QM9414 (Merk – Germany)
- ✓ *Saccharomyces cerevisiae* strain ATCC-42368

Substrates

Corn cobs, beech shavings (of Brasov County), fir shavings (of Brasov County)

Methods

Preparations

The lignocellulosic residues were processed, made of 1 – 1.5 kg batches, with an average density of 0.2 – 0.7 gr/v.

Corn cobs (after removing the corn grains), dried by warm air ventilation, with an average contents of dry substance of 88.92% were chopped in a mill until the particles` sizes reached 20 to 40 meshes. The beech chips and the fir ones, the result of the beech or of the fir trees` filling for various purposes (for either timber or furniture production) was dried and chopped in the same fashion^{1,2}.

The residues were further introduced into the autoclave, in a ratio of 1:10, dry residue: sulfuric acid 4% g/v, at 1.5 atm for 45 minutes.

The acid hydrolysis was performed with sulfuric acid, therefore separating the liquid fraction from the residue, using a screw press, after which the raw hydrolyzate was introduced into a centrifugal separator, in order to separate the particles of the suspension. The clear filtrate was then neutralized with calcium carbonate, through intermittent agitation.

The purification and the concentration of the acid hydrolyzate was performed by removing the calcium sulfate suspension by filtering and, thus, a liquid fraction was obtained, that mainly contains the sugars of the pentosan hemicelluloses, with a concentration in between 1.7% and 4.2%, according to each substrate, and with a dry substance content of 3 to 7%^{2,5}.

The calcium sulfate is removed by filtering, thus obtaining a liquid fraction which contains the sugars of the pentosan hemicelluloses, with an average content of 1.7% to 4.2% (according to each substrate) and with a dry substance content of 3%-7%.

The solid residual fraction that resulted after the hemicelluloses` hydrolysis was washed in the filter, in order to remove the hydrolytic agent, and until the pH value reached 4.8.

The solid residual fraction is mainly composed of the lignocellulosic residue (cellulignin) with a variable degree of destructuration, quite convenient to be candied by treating with celluloso – lytical enzymes.

The liquid fraction was then concentrated using two methods: evaporation in vacuum and microwave concentration. In both cases a 5% to 15% concentration of dry substance was obtained, while the reducing sugars were in between 4% to 8%.

The concentrated hydrolyzate was then purified by adding active coal 0.3%, considering the liquid phase`s volume, with maintaining it at the room`s temperature for about 2 – 3 hours, continuously agitating. Later, the

filtration phase followed, which led to a liquid with „0” transparency, while the sediment mainly kept the phenolic compounds and some colored substances, the result of some compounds` degradation throughout the acid hydrolysis phase. At a sulfuric acid concentration of only 4% g/v the degradation level is not significant.

I. The pentosan sugars` fermentation

The filtrate was submitted to fermentation, using the *Pachysolen tannophilus* yeast, strain CBS-4044 NRRLY-2460. The fermentation was performed with both microaeration and continuous agitation, at 240 rpm, in a bioreactor at 28°C for 72 hours. The densities of the yeast cells of the *Pachysolen tannophilus* was $1,5 \times 10^8$ /ml per inoculum. The *Pachysolen tannophilus* yeast was used, that has the ability to perform the bioconversion to ethanol of pentosic sugars and of some derivatives. The bioconversion yield, evaluated with xylose as a standard, was of a 30,6% average³.

Distillation of the ethanol obtained from the pentosanic sugars

The gathered ethanol was after distilled and corrected according to the classical procedure, therefore a mash was obtained, in which proteins, macro and micro mineral nutrients, lipids, vitamins and some volatile compounds are found. The remaining solid residue, after the pretreatment phase was introduced into a bioreactor and further treated with an acetate buffer solution to a 4.8 pH. The ratio: solid residue:buffer was 1:7. The cellulosic residue, the result of the pressing operation, was of 26 to 27% humidity.

II. The enzymatic hydrolysis of the lignocellulosic substrate

The cellulolytic product was added, obtained before in our own laboratories. Therefore, the ICA 3196 *Trichoderma viridae* strain was used, that produced enzymes with the specific activity of 20.000 - 22.000 FPU/kg residue dry substance. The enzymatic hydrolysis was performed at 50°C, at a pH=4.8 for 40 hours, at 200 rpm. The enzymatic hydrolysis was performed with an enzymatic concentrate prepared in the Institute of Food Chemistry (ICA) laboratories, with 21.42 FPU/ml enzymatic product, and 1 FPU was used for treating 46.68 mg of lignocellulosic substrate. The added quantity of enzymatic product was calculated considering the substrate`s mass, in FPU/kg dry residue units⁴.

The cellulolytic product came from *Trichoderma viridae* (ICA), strain 3196, cultivated onto the potato dextrose – agar medium, after growing it

for 10 days at 30°C.

The carbon source is the filter paper. The nitrogen source is a peptone enriched with urea and with macro and microelements (Mg, Ca, Fe, Zn, Mn).

The culture medium was concentrated in vacuum, using Simax equipment, thus obtaining a cellulolytic concentrate with an activity of 10.2 FPU/ml – 21.42 UPF/g. For 5g of residual substrate, 10.5 ml of enzymatic concentrate was added, that is the equivalent of 107.1 FPU units.

In a similar fashion the experiments with the *Trichoderma reesei* QM9414 (Merk-Germania) were carried out.

Alcoholic fermentation of the enzymatically hydrolyzated cello lignin

After the enzymatic hydrolysis, the liquid fraction, separated by pressing does contain glucose and its oligomers. Further, the solution of sugars is to be concentrated in vacuum or with microwaves to half its initial volume. The concentrated fraction was submitted to fermentation, using the classical technique and the *Saccharomyces cerevisiae* strain ATCC-42368 yeast, at 30°C.

Determining the ethylic alcohol

The ethylic alcohol concentration was around 2% - 2.3%.

The bioconversion yield was in between 28% to 32%. Then, the distillation and the correction of the gathered ethylic acid took place. At the same time, a mash was obtained which contains the yeast biomass, along with proteins, phenolic compounds, vitamins, residual carbohydrates and others. The mash, as a residual waste of the ethylic alcohol distillation process, was dried up to 80% dry substance.

The distillation and the correction was performed through bringing together, in the same installation the ethanol that was the result of the hemicellulosic pentosans` bioconversion and the one that was the result of the cellulolytical saccharification. By drying the mash with concentrated solutions of sodium hydroxide 2N, the remaining lignin throughout the lignocellulosic structures was separated through saponification and filtration, further submitted to acidification to release the Na⁺ ion as a salt and finally extracted with organic solvent, with ethyl ether or ethyl acetate.

Through the distillation of the organic phase a brown powder was collected, with a variable content of oligophenols. Determining the phenolic compounds was performed out of the liquid fraction of the hydrolyzate after the sulfuric acid was neutralized with calcium carbonate, up to a pH of 5.4 – 5.8, and after separating the liquid phase from the remaining organic residue and from the calcium sulphate precipitate.

Evaluating the total phenols was performed by the Folin – Ciocâlteu technique [1, 2, 5], at a wavelength of 765 nm (standard – galic acid) as shown in Table 3.

After separating, by pressing, the lignocellulosic residues of the oligophenolic structures solubilized in organic solvents, the remaining biomass shows a microporous structure with potential applications as a natural fertilizer, for improving the degraded soils or as insulation biomass^{1,5}.

RESULTS AND DISCUSSION

The percentages of reducing sugars, the hemicellulosic ones, released by the pretreatment phase, at a hydromodule of 1:10 lignocellulosic substrates: sulfuric acid 4% g/v are detailed in table 1.

Table 1: Fermentable sugars distribution, in various lignocellulosic substrates, in the pretreatment phase

Features	Beech chips	Fir chips	Corn cobs
Reducing sugar, % of the lignocellulosic	18.7 - 20.7	14 - 16	33 - 38.5
Reducing sugar, % in the initial liquid fraction	1.3 - 1.7	0.9 - 1.05	3.5 - 4.2
Reducing sugar, % after microwave treatment	3.8 - 4.5	2-2.2	7.8 – 8.2

Experimental data regarding the levels for the total amount of sugars and for the quantity of reducing sugars are displayed in figures 1 and 2.

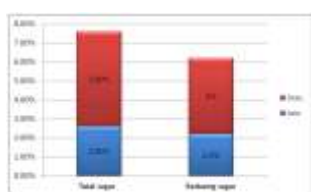


Fig.1. Total sugars level and reducing sugars level after enzymatic saccharification

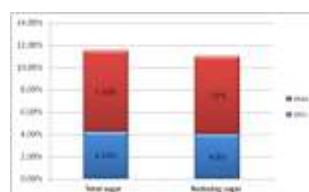


Fig.2. Total sugars level and reducing sugars level after vacuum concentration

After the *Saccharomyces cerevisiae* yeast, strain ATCC-42368 fermentation of the solution of sugars, the concentration in ethylic alcohol was in between 2 and 2.3%. The bioconversion yeast, of sugars from the enzymatic cleavages of the beta – glycozidic bonds from the lignocellulose into ethanol, the values were in between 28-32%.

The liquid fraction of the pretreatment phase, performed with sulfuric acid 4% g/v, separated from the lignocellulosic residues contains the sugars from heteropolysaccharides and the phenolic oligomers and represent 10-18% of the total amount of lignin from the initial material. Therefore, the UV absorption levels of the mentioned hydrolyzates reaches values of 0.62 – 1.2. The oligophenols that can be recovered out of the heteropolysaccharidic hydrolyzate are 2.5 to 4.5% of the whole amount of dry substance.

Compared hydrolysis

Table 2 shows the amounts of sugars that were the results of the enzymatic treatment with the ICA cellulolytic product, in comparison to the *Trichoderma reesei* QM9414 (Merk – Germany) one, with an enzymatic activity declared of 1U/mg.

One unit of cellulosic activity is the quantity of enzyme which releases 1 μ M of reducing carbohydrates per one minute (DNS method with 3,5 dinitrosalicylic acid), in the following conditions, of a pH = 4.5, at a temperature of 45⁰C).

Table 2: The concentration of the fermentable resulted in the enzymatic hydrolysis, when using the I.C.A. enzyme, in similar conditions to the enzymatic product of *Trichoderma reesei* (QM 9414)

Substrate	Enzymatic product	Reducing carbohydrate (mg/ml)
Beech shaves, Brasov County	<i>Trichoderma reesei</i> QM9414	6.4
Fir shaves, Brasov County	<i>Trichoderma reesei</i> QM9414	43
Beech shaves, Brasov County	Celluloso – lytical Product I.C.A.	6.1 – 6.7
Fir	Celluloso – lytical Product ICA.	3.9-4.9

shaves, Brasov County		
Corn cobs	<i>Trichoderma reesei</i> QM9414	22.5
Corn cobs	Celluloso – lytical Product ICA.	14.4-16.2

The tested I.C.A. cellulolytical product is *Trichoderma viridae*, strain 3196 ICA³.

The values of the global phenols, solubilized in aqueous medium, expressed in percentages (%) of the mixture`s dry substance are shown in Table 3.

Table 3: The total phenol content, released after the fungal attack took place

Experimental version	Total phenols (% of d. S.)
Beech shaves, Brasov County + <i>Trichoderma reesei</i> QM9414	3.46
Fir shaves, Brasov County + <i>Trichoderma reesei</i> QM9414	4.02
Corn cobs + <i>Trichoderma reesei</i> QM9414	2.82
Beech shaves, Brasov County + celluloso – lytic product of I.C.A	3.14
Fir shaves, Brasov County + celluloso – lytic product of I.C.A	3.84
Corn cobs + celluloso – lytic product of I.C.A	2.56

CONCLUSIONS

The sulfuric acid 4% g/v pretreatment was performed for a hydromodule of 1:10, for 45 minutes at 1.5 atm. In these conditions, from the beech chips and from the corn cobs, sugars in concentrations mos adequate for bioconversion to ethanol, in the yeast fermentation of *Pachysolen tannophilus* are released, with yields of about 30.6%, (reported to xylose).

The enzymatic hydrolysis with the ICA product, at a concentration of 21.42 FPU/g substrate is comparable to the one performed with *Trichoderma reesei* QM9414 (Merk – Germany), in similar enzymatic processing conditions.

Considering the bioconversion technologies of the lignocelluloses currently applied, the technique submitted by the present paper is to be easily accomplished using materials and equipments quite accessible (as presented by Scheme 1), for when they are turned into account in an integrated manner, along the fermentable sugars and the phenolic compounds with

high antioxidant potential and with the proteic biomass of the mash. The submitted version offers the possibility of a sequential turning into account of the hemicellulosic polysaccharides into ethylic alcohol, along with the recovery of the polyphenols with antioxidant potential. The recovery level for the phenolic structures with high antioxidant potential is in between 2.5% - 4% of the dry substance for the processed residue. The obtained fermented product is substantially enriched with organic nitrogen to be used as natural fertilizer in order to remedy degraded soils, or even as insulation biomass.

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