SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF TOBACCO SAMPLES

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ABSTRACT

Tobacco plants *(Nicotiana rustica, Nicotiana tabacum)* produce abundant biomass in more than 100 countries and could be used to produce abundant biofuels. Tobacco is an ideal crop for biobased products; it is a perennial herbaceous plant. About one quarter of the tobacco plant is cellulosic material. Tobacco stem is a by-product in the tobacco industry after the tobacco leaves are harvested. The utilization ratio of tobacco stem is only approximately 40% due to difficulties in the comprehensive utilization technologies of tobacco stem. Therefore most of the tobacco stems have gone to waste. This biomass appears attractive for conversion to ethanol because it contains very low amounts of the hard-to-convert woody material lignin. The aim of this study is to identify the best parameters of the simultaneous saccharification and fermentation (SSF) process on tobacco based substrate. Just to make this technology cheaper, the enzyme recovery was investigated by membrane technology. Polyethersulfone membrane filters with 0.1 μm were used for microfiltration followed by determination of proteins using photometer and Kjeldahl method to measure the enzyme recovery. The aim was to decide whether the microfiltration membrane with cut-off 0.1 μm is a proper membrane for recycling the enzyme. During the filtration, increased resistance and decreased flux were detected.

Keywords: tobacco, biofuel, microfiltration

1. INTRODUCTION

With the inevitable depletion of the world's petroleum supply [11], there is an increasing worldwide interest in alternative, non-petroleum-based sources of energy. As petroleum supplies 97% of the energy consumed for transportation [18], industry and governments worldwide have been actively identifying, developing and commercializing technology for alternative transportation fuels over the past 20 years [20, 24]. Due to diminishing fossil fuel reserves, alternative energy sources need to be renewable, sustainable, efficient, cost effective, convenient and safe [5].

Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars [3,10,24]. Ethanol is an attractive alternative fuel since that it can be blended with gasoline or used as neat alcohol in dedicated engines, taking advantage of the higher octane number and higher heat of vaporization [9]. Currently, Brazil and the United States are the two countries that produce large quantities of fuel ethanol from sugar cane and maize respectively, and they together account for about 70% of the world bioethanol production [4].

Nicotiana spp. is one of the most important nonfood crops that are widely cultivated worldwide [16]. As a raw material, tobacco is used to yield smoked food-tobacco products, which are intended to be smoked, sucked, chewed or snuffed. However, the health risk of tobacco consumption is getting more and more attention by people. For these years, in order to reduce harmful component of cigarette smoking, the biomass material of tobacco stem have been widely applied to cigarette, such as expanded stems [20], reconstituted tobacco sheets [7]. Tobacco stem is a by-product in the tobacco industry after the tobacco leaves are harvested. Unfortunately, the utilization ratio of tobacco stem is only approximately 40% due to difficulties in the comprehensive utilization technologies of tobacco stems. Therefore, most of the tobacco stems have gone to waste. According to an incomplete statistical analysis of 2008, more than 366.2 thousand tons of tobacco stems were disposed of as rubbish worldwide. It led to enormous waste of natural plant resource and serious environmental pollution [12], [13] [24]. Therefore, the reutilization of this industrial waste and the exploitation for potential biomass material would be important and indispensable [19] [21] [25]. Processes based on membranes have been suggested as a good solution for the enzyme recovery because they are energy efficient processes and provide an efficient treatment [8][22].

2. MATERIAL AND METHODS

2.1. Raw material

The hydrolyzate was made from tobacco "Experimental" and "By-products" tobacco samples and they were get from a Hungarian tobacco plant cultivation. The "experimental" (EX) samples were the whole plant, the stem and leaves at all. Meanwhile the "by-product" (BY) consisted mainly on the stem, the part of plant after tobacco-processing. The samples were cut and frozen after harvesting immediately and were keeping in deep frozen until hydrolysis. One part of the samples was cut by cutter to reduce the size of particles before hydrolysis.
Dry matter (DM) was determined by drying the samples overnight at 105 °C.

2.2. Enzymatic saccharification

Saccharification of the samples was carried out in a 2 L fermentation unit (Labfors Minifors, Belgium) at 30°C±0.2 and pH 4.5±0.1 at 160 rpm.
The substrate concentration in the glass flask was 80 g DM/l. The enzyme (endo-1,4-β xylanase from *Trichoderma longibrachiatum;* Sigma Aldrich) and the yeast *(Saccharomyces cerevisiae,* Unikén wine yeast) dose was determined by experimental design that is why it was applied dosed in a different concentration of 4000 or 4800 mg/ enzyme and 1000 or 1500 mg of yeast. The glucose release was monitored after 1, 24, 48, 72 and 96 hours.

2.3. Sugar content

The sugar content was determined spectrophotometrically using 3,5- dinitrosalicylic acid (DNS) method, after calibration. This method tests the presence of free carbonyl group (C=O), called reducing sugars.
It involves the oxidation of the aldehyde functional group present, for example, in glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions because dissolved oxygen can interfere with glucose oxidation. Sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen [15].
All samples were diluted 10 times and subsequently 300 µl of DNS were added to 300 µl of samples. The mixtures were heated at 90 °C for 10 minutes to develop the red-brown color. After the heating, 100 µl of potassium sodium tartrate (Rochelle salt) was added in all samples, thereafter the samples were put in a cold water bath and the absorbance was recorded with a spectrophotometer (Nanocolor UV/VIS, Macherey-Nagel) at 540 nm [14,15].
The sugar content was measured at the received ferment-juice and it was given per unit dry material weight basis.

2.4. Centrifuge

The fermented liquid samples were centrifuge at 10 minute and 6500 RPM.

2.5. Ethanol content

Analyses of the ethanol content of the centrifuged fermented liquid was performed at 153°C for 30 minute , in gas chromatograph (DANI Master Restek) with Stabilwax column, which is 30 m long, diameter is 0.25 mm and the film thickness is 0.5 µm. Hydrogen was used as a carrier gas.

2.6. Microfilter equipment

Separation was carried out by a stirred cell device with capacity of 400 cm^3 equipped with a 0.004534 m^2 polyether-sulfone (PES) membrane with a MWCO of 0.1 µm. The sample was mixed continuously with a magnetic stirrer during separation. The relevant data on the membranes are presented in Tab. 1.

Table 1. Characteristics of membranes used

Membrane	Maximum pressure [bar]	MWCO [g mol^{-1}]	Maximum temperature [°C]	Recommended pH Range
PES	0.5-5	0.1 µm	90	2-11

The selectivity of a membrane for a given solute and the efficiency of the process were expressed by the retention (R):

$$R = \left(1 - \frac{c}{c_0}\right) \cdot 100 \quad (\%) \tag{1}$$

Where c is the concentration of the permeate phase (% or mg dm^{-3}), and the c_0 is the concentration of the feed (% or mg dm^{-3}).
The permeate flux (J) can be described as a function of time [6, 8]:

$$J = \frac{dV}{dt} = J_0 t^{-K} \quad (\mathrm{Lm^{-2}h^{-1}}) \tag{2}$$

Where J_0 is the initial permeate flux (L m^{-2} h^{-1}), t is the filtration time (h), and K is the fouling index.
The membrane resistance (R_M) was calculated as:

$$R_M = \frac{\Delta p}{J_w \cdot \eta} \quad (\mathrm{m^{-1}}) \tag{3}$$

Where J_W is the flux of water (m^3 m^{-2} h^{-1}), and η is the water viscosity (Pas).
The fouling resistance (R_f) of the membrane can be measured after washing the gel layer from the membrane. R_f and the resistance of the gel layer (R_g) can be calculated as:

$$R_f = \frac{\Delta p}{J_{WW} \cdot \eta} - R_M \quad (\mathrm{m^{-1}}) \tag{4}$$

$$R_g = R_t - (R_M + R_f) \quad (\mathrm{m^{-1}}) \tag{5}$$

Where J_{WW} is the flux of water (m^3 m^{-2} h^{-1}) after gel layer removal and J_F is the flux of fermented liquid (m^3 m^{-2} h^{-1}).
Reynolds' number in the case of mixing can be calculated via the equation.

$$\mathrm{Re}_{mix} = \frac{d^2 n \rho}{\eta} \quad (-) \tag{6}$$

Where ρ is the density (kg m^{-3}), n is the rotation rate of the stirrer (s^{-1}), η is the viscosity (Pas), and d is the diameter of the stirrer (m).

2.7. Protein content

The protein quantity was determined by the Kjeldhal method. The method consists of three steps: 1) digestion of the sample in sulphuric acid with a catalyst. The nitrogen contained in the sample is converted to ammonia; ammonium sulphate being formed. 2) Distillation of ammonia released from ammonium sulphate by addition of an excess of sodium hydroxide; ammonia being trapped in a trapping solution (sulphuric acid). 3) back-titration of the excess of the trapping solution.

The percentage of crude protein (CP) can be found by multiplying the percent Nitrogen by a factor (usually 6.25).

$$CP = \%N \cdot 6.2 \tag{7}$$

The second method for determination of protein content was spectrophotometrically. The absorbance of samples was measured at 280 nm. The average molar extinction coefficient of proteins is $1.6*10^5$ mol/cm. Distilled water was reset and using the Lambert-Beer law to determine the concentration:

$$A = \varepsilon \cdot c \cdot L \tag{8}$$

Where A is the measured absorbance at 280 nm, ε is the molar extinction coefficient, c is the concentration, L is the pathlenght of the light in cm (in this case the thickness of the cuvette). Molar extinction coefficient (ε) is a measurement of how strongly a chemical species absorbs light at a given wavelength.

3. RESULTS AND DISCUSSION

The aim of the work was to determine optimum parameters of the enzymatic hydrolysis of cellulose to monosaccharides by factorial experimental design. In the first part of this work, a factorial experimental design whit using of excel spreadsheet was prepared. [17].
Tab. 2 shows the experimental data of EX and BY. In the table can be seen that the amount and rate of the xylanase and yeast was changed only at the experiments. One part of the samples were cut by cutter to reduce the size of particles before hydrolysis, therefore the effect of the size reduction was investigated also.

Table 2. Experimental design data of the EX and BY

Samples	H_20 [mL]	Xylanase [mg]	Yeast [mg]	Substrate weight [g]	Measured dry weight [g]	pH	T [ºC]
EX	1000	4000	1000	80	0.586	4.5	30
EX	1000	4800	1500	80	0.586	4.5	30
BY	1000	4000	1000	80	0.513	4.5	30
BY	1000	4800	1500	80	0.513	4.5	30

The Fig. 1-2 and 3-4 demonstrates the effect of the reaction time on the enzyme hydrolysis and ethanol yield at the minced EX and BY tobacco. The results show that the best reaction time for enzymatic hydrolysis of lignocellulose is 24 hours. Optimal enzyme parameters to reach the highest sugar concentration was 4800 mg for the EX samples and 4000 mg for the BY samples.

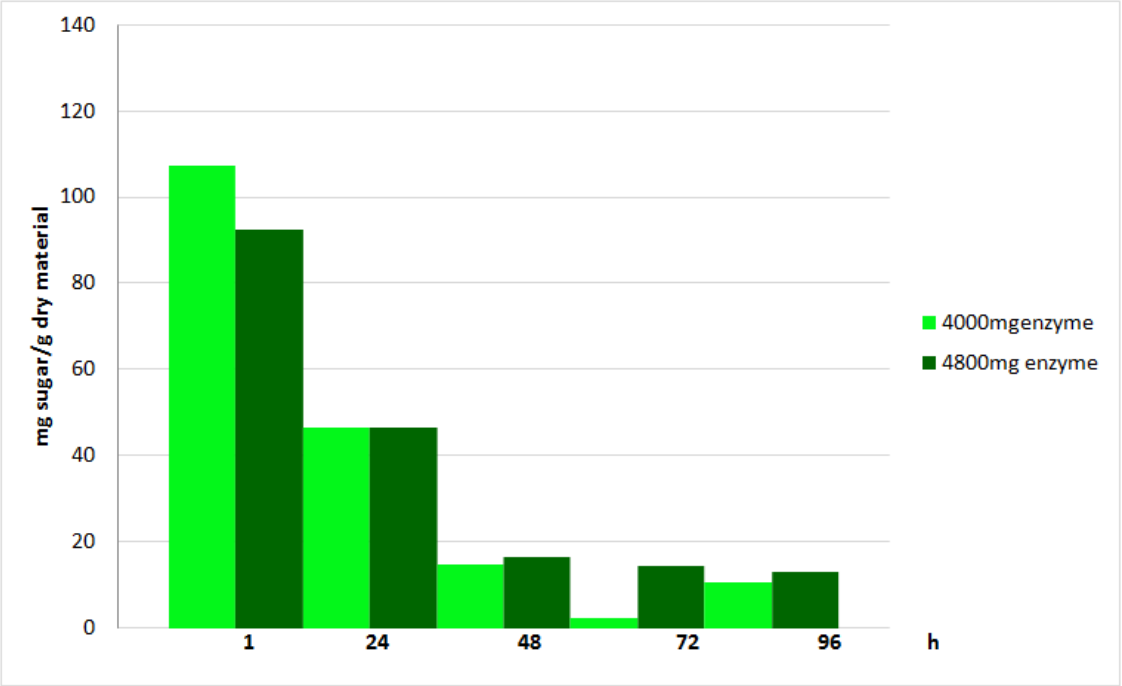

Figure 1. Effect of the reaction time on the enzyme hydrolysis of minced EX tobacco

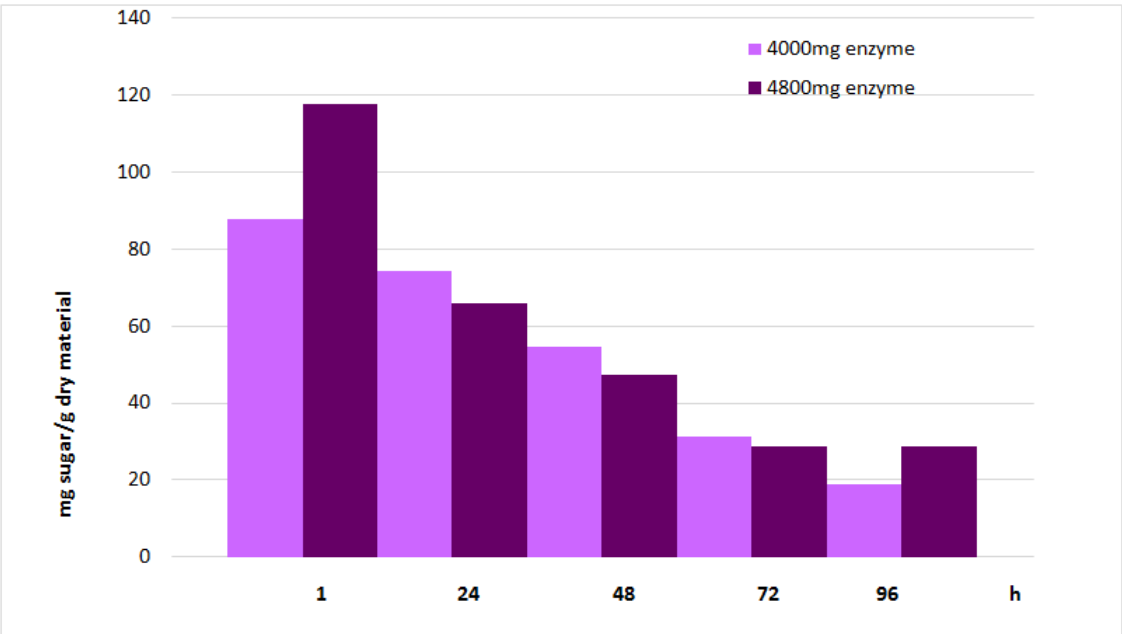

Figure 2. Effect of the reaction time on the enzyme hydrolysis of minced BY tobacco

Ethanol content of each samples were measured on GC after 1; 24; 48; 72 and 96 hours. Fig. 3 and 4.shows the ethanol yield at the By-products (BY) and at the Experimental (EX) tobacco. The optimal time and yeast parameters of the EX samples was 48 hours and 1500 mg yeast and 48 hours 1000 mg yeast at the BY samples. The ethanol concentrations were not so high that is why it will be optimized in the future.

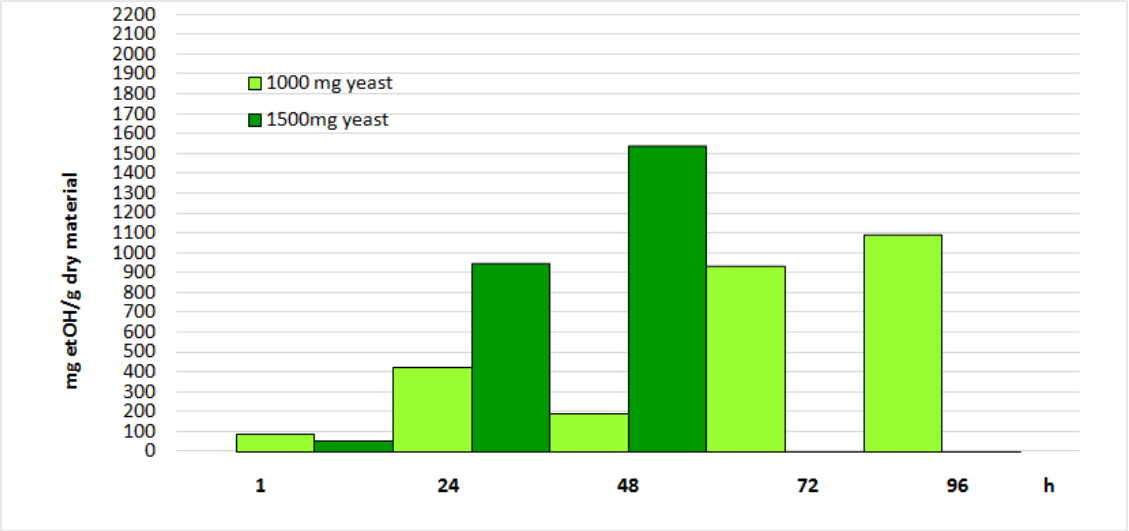

Figure 3. Ethanol yield at Experimental tobacco

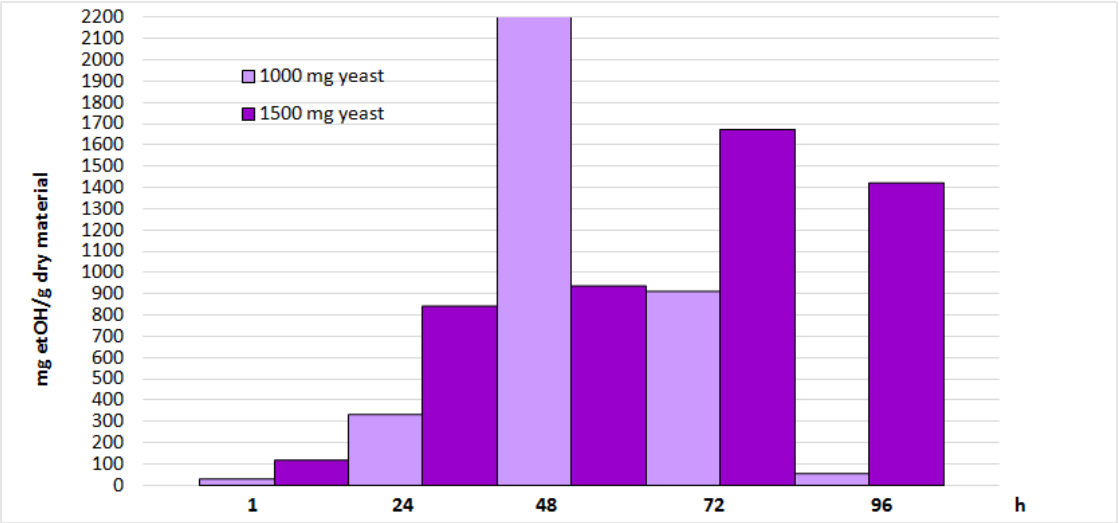

Figure 4. Ethanol yield at By-product tobacco

The second aim of the work was to investigate the possibility of enzyme recovery. For this, the membrane separation was made and the most important separation parameters were determined.
The initial flux of the model solution was the highest due to the composition of the hydrolysate of EX and BY that covered a very wide range of molecules, and the model solution consists only of disaccharides and enzymes (Fig. 5). The flux decline is the highest at the model solution also what is shown by the fouling index values and in the Fig. 6 as well.

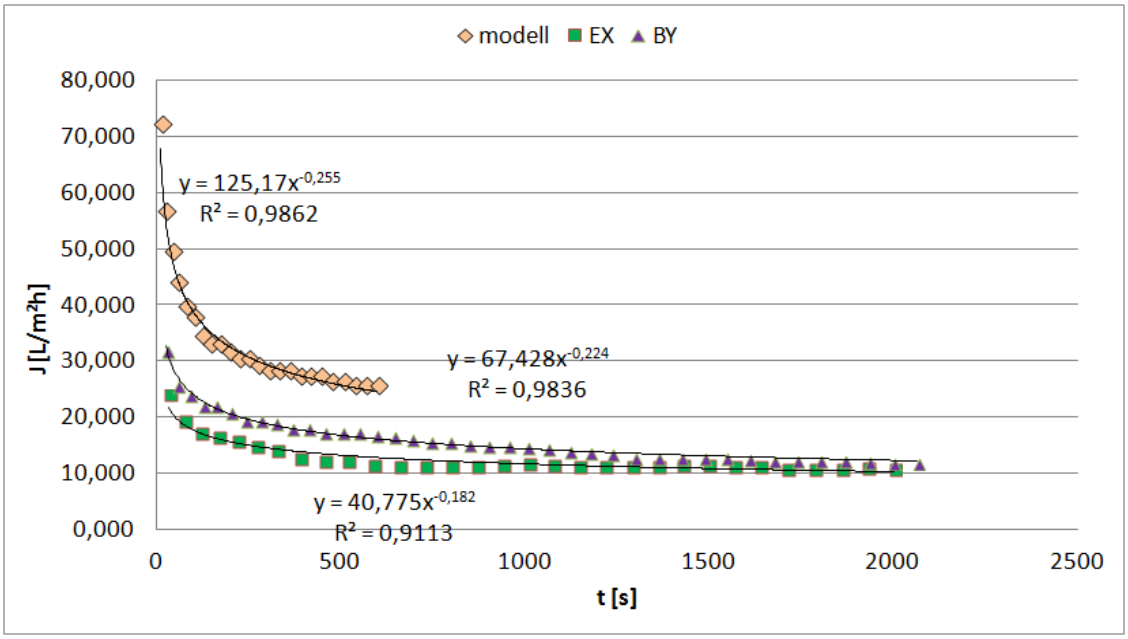

Figure 5. Flux values of the different tobacco samples

There is a significant difference between the curve of model solution and of tobacco hydrolysate samples. The amount of components contained in the hydrolysates makes this difference. There is only a slight difference between the fermented liquid. It shows the difference of the originating, i.e. the BY samples have relative more poorly degradable cellulose fibers than the EX samples.

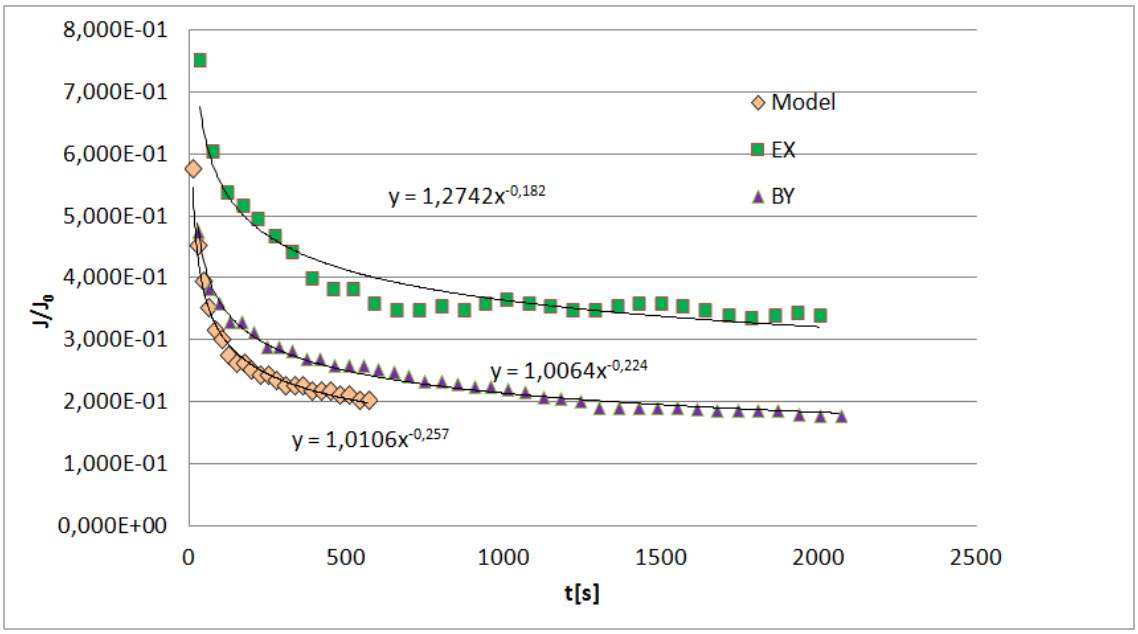

Figure 6. Relative flux values of the different tobacco samples

Three different membrane resistance values were measured and calculated (equations 3-5) during the experiments. First the membrane resistance (Rm), second the resistance of the gel layer on the surface of the membrane (Rg), and finally the fouling resistance (Rf).

Fig. 7 shows that the resistance values were the biggest for the EX samples. Probably it can be concluded that the enzymatic hydrolysate of the cellulose content of EX contains several smaller fragments than the

BY samples. These small particles can penetrate into the membrane pores and increase the fouling resistance. The EX samples contain the whole plant, not only the stem and petiole, so the EX samples have specifically less thick cellulose fibres.

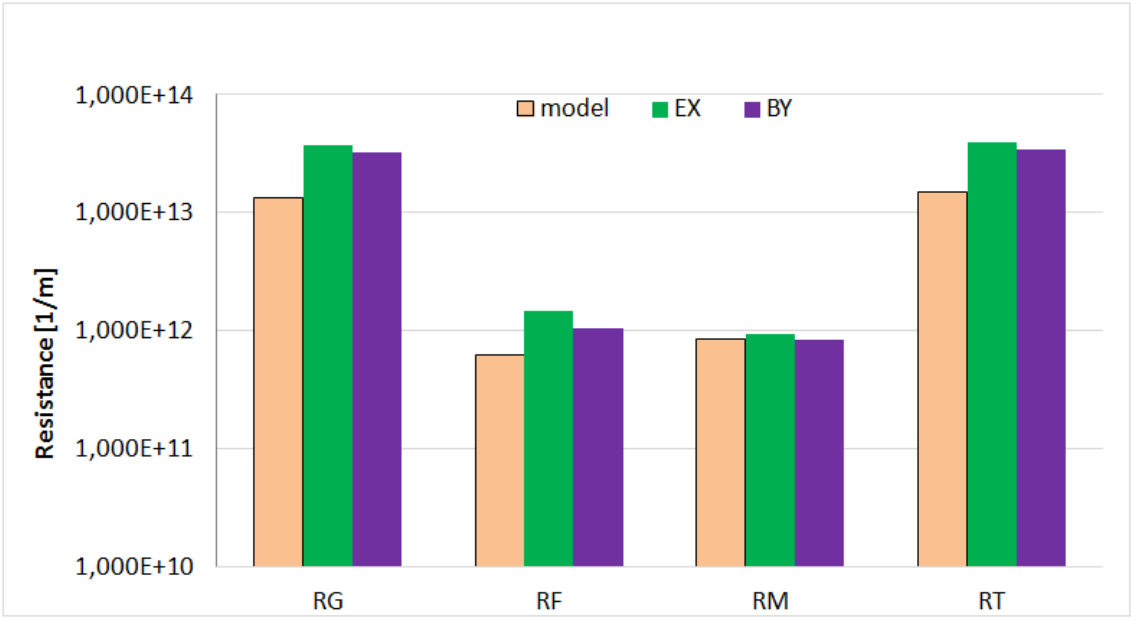

Figure 7. Resistance values of the different tobacco samples

The protein retention values in Fig. 8 shows that the enzymes/ proteins could be separated into the concentrate and also how the 0.1 µm PES membrane retains the proteins/enzymes. Here it can be seen that the value of the proteins in the model solution is the best, but the value of the EX samples is higher than the BY samples, while the composition of the hydrolysate covered a very wide range of molecules, and the model solution consists only of disaccharides and enzymes.

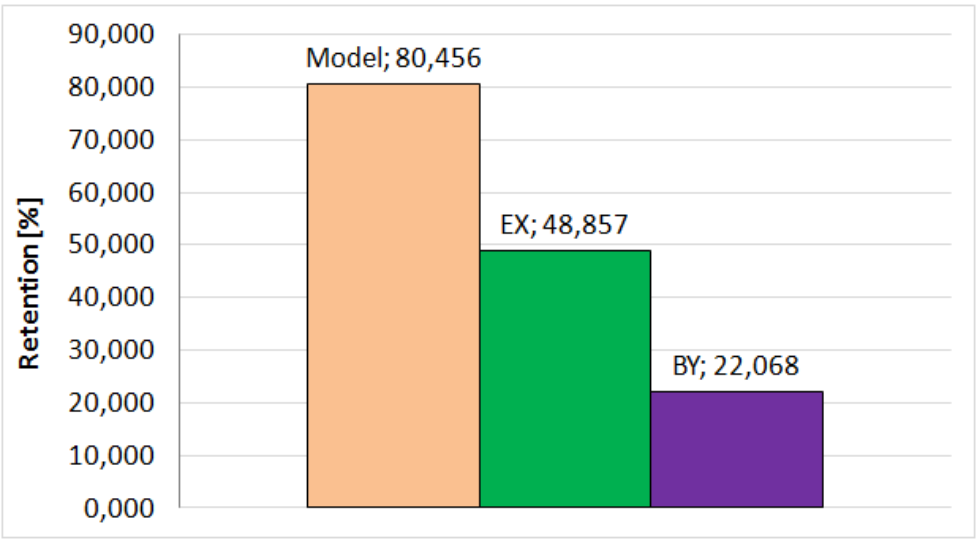

Figure 8. Protein retention values of the different tobacco samples

4. CONCLUSION

In this work the utilization of organic waste from tobacco processing was examined in ethanol production. The main aim of this study was to examine and intensify the enzymatic hydrolysis of cellulose to monosaccharides in order to obtain higher ethanol yield. The bioethanol yield can be large-scale increased by the optimized condition of cellulose hydrolysis prior to the fermentation.
In these experiments the ratio of enzymes to substrate and the reaction time were varied to find the best condition of hydrolysis. The amount of hydrolysed sugar was determined from EX and BY tobacco samples. In both experiments the glucose concentration is high in the samples at 1 hour and 24 hours. The ethanol content of the fermentation broth was determined by gas chromatography. The results showed that the ethanol yield of the EX samples was better at 48 hours with 1500 mg enzyme but at 72 hours and 96 hours with 1000 mg enzyme content samples showed better values. In the case of BY samples there was higher ethanol yield at 48 hours with 1000 mg enzyme and 72; 96 hours with 1500 mg enzyme. Our preliminary results showed that tobacco is a great source of renewable materials that is why it could become a useful biofuel feedstock.
This study shows that the membrane filtration with 0.1 μm cut –off value membrane could help recovering the enzyme, which was used in the fermentation of tobacco samples to create bioethanol. More measurements are needed to check the real enzyme content of the permeate.

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