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## COMBINED PRE-TREATMENT FOR SACCHARIFICATION

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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 since about one quarter of the tobacco plant is cellulosic material [1]. This biomass appears attractive for conversion to ethanol because it contains very low amounts of the hard-to-convert woody material lignin [1]. Microwave (MW) irradiation has a good potential to increase the ability of organic matter for biological degradation so we connected the enzymatic hydrolysis of the cellulose type material of the tobacco plant and the microwave pretreatment. Our results show that microwave pre-treatments was suitable to enhance the biodegradability of tobacco plant and this way the MW pre-treatment was suitable for enhancing the sugar yield. The saccharification time was shortened by the alkali pre-treatment so the combination of alkali pH and MW could be the better solution. The objective of our work was to investigate how to affect the MW pre-treatment on the enzymatic hydrolysis.

**Keywords:** microwave; saccharification; tobacco; bio-fuel, by-product

### INTRODUCTION

Tobacco plants (*Nicotiana rustica*, *Nicotiana tabacum*) is an ideal crop for bio-based products it is a perennial herbaceous plant. It is found only in cultivation where it is the most commonly grown of all plants in the *Nicotiana* genus and its leaves are commercially grown in many countries to be processed into tobacco. It grows to heights between 1 to 2 meters [1]. Tobacco is sensitive to temperature, air, ground humidity and type of land. Temperatures of 20-30 °C are best for adequate growth, an atmospheric humidity of 80 to 85% and soil without a high level of nitrogen are also optimal [1]. Tobacco has potent oil biosynthesis machinery, which produces up to 40% oil per seed dry weight [2]. Recently, tobacco seed oil has been successfully tested for its potential as a fuel for diesel engines [3]. Tobacco leaves contain 1.7%–4% oil per dry weight [4], which is extractable as fatty acid esters, the major component of biofuel oil [5]. Cultivation of tobacco for biomass is very different from conventional tobacco. While conventional tobacco production is labor-intensive, biomass tobacco is largely mechanized. Thus, production costs are substantially reduced. The plants are grown much more closely together and for biomass tobacco it is possible to obtain multiple harvests in a single season from a single crop of tobacco, the plants will re-grow following a harvest [5]. Tobacco produces more biomass than virtually any other agricultural crop. Tobacco naturally produces large volumes of starches and sugars [6].

Lignocellulose-containing waste is very common for example in tobacco industry. The main problem with the lignocellulose is the necessity of hydrolysis before fermentation since it cannot be transformed directly into bioethanol. First it should be transformed (with hydrolysis) into glucose and after the glucose should be fermented into ethanol [7]. It is also possible to do these two processes in the same time: it is called

Simultaneous Saccharification and Fermentation (SSF) [8]. Different ways had been tested to make the cellulose more accessible for fermentation. Pre-treatment greatly affect the efficiency of saccharification and the ethanol production cost as well. Over the years, a number of different methods such as steam explosion [9], thermal methods [10], acid and alkaline hydrolysis pre-treatment has been developed to enhance the cellulose degradation, remove hemicellulose and lignin and alter the structure of them. Microwave heating is based on the ability of a particular substance such as a solvent or substrate to absorb microwave energy and effectively convert the electromagnetic energy to heat. Molecules with a dipole moment attempt to align themselves with the oscillating electric field of the microwave irradiation, leading to rotation. In liquid and solid phase, this rotation produces friction which results in an increase of the temperature. It is possible to achieve rapid and uniform heating of relatively thick materials. When microwave are directed towards a material, part of the energy is reflected, part is transmitted through the surface and of this latter quantity, and part of it is absorbed [11].

Although the use of microwave for cooking is widespread, the application of this technology to the processing materials is relatively new development. As many researchers have already stated in numerous published papers microwave irradiation (usually at the ISM – Industrial Scientific and Medical – frequency of 2.45 GHz) produces efficient internal heating for most chemical reactions, delivering energy exactly where it is needed, even under exothermic conditions. Another valuable advantage of using controlled microwave dielectric heating for chemical synthesis is the dramatic reduction in reaction times: from days and hours to minutes and seconds. These two properties are sufficient

motivation to promote the use of microwaves in “greener chemical processes.” [12].

The aim of this study is to examine the effect of MW pre-treatment on the enzymatic hydrolysis of cellulose, to optimize the process parameter of MW pre-treatment and to investigate the efficiency of simultaneous saccharification and fermentation (SSF).

## MATERIAL AND METHODS

### Raw material

“Experimental” and “By-products” tobacco samples were get from 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.

### Enzymatic saccharification

The saccharification of tobacco samples were made in a 2 liter laboratory fermentation unit (Labfors Minifors, Belgium) [13]. For enzymatic hydrolysis cellulase (CLA) (Cellulast 1.5L, Novozymes A/S, Denmark; 700 U/g) from *Trichoderma reesei* (Sigma-Aldrich) and cellobiase (CLB) (Novozym 188, Novozymes A/S, Denmark; 250 U/g) from *Aspergillus niger* (Sigma-Aldrich) was applied dosed in a different concentration of CLA 14.88 cm<sup>3</sup>/L (EX), 18.64 cm<sup>3</sup>/L (BY) and CLB 18.32 cm<sup>3</sup>/L (EX), 15.44 cm<sup>3</sup>/L (BY). The temperature and pH of enzymatic hydrolysis were controlled at 50±0.2°C and pH 5.0±0.1.

### Sugar content

The sugar content was determined spectrophotometrically with using of the 3, 5- dinitrosalicylic acid (DNS) method, after calibration. This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, 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, sulphite, which itself is not necessary for the colour reaction, is added in the reagent to absorb the dissolved oxygen [14]. 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 colour. After the heating, 100 µl potassium sodium tartrate (Rochelle salt) was added in all samples and 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]. The sugar content was measured at the received fermentation broth and it was given per unit dry material weight basis.

### Conductive heat pre-treatment

For the conductive heat treatments a hot-plate magnetic stirrer (ARE Heat Magnetic Stirrer) was used. The treatment was carried out at 85 °C and the temperature was kept controlled by electronic contact thermometer.

### Alkaline pre-treatment

The alkaline pre-treatment was performed with thermal treatment too. This type of treatment was different from the main thermal treatment because in this case of the pH of the treated samples was adjusted to 10 with 0.1 M sodium hydroxide solution.

### Microwave pre-treatment

Microwave pre-treatments are carried out in Labotron 500 (Bucher-Guyer AG) professional laboratory microwave equipment. Labotron 500 laboratory microwave unit operated with output power of 250 W and 500 W 2450 MHz frequency [15].

## RESULTS AND DISCUSSION

The purpose of the pre-treatment is to make the lignocellulosic structure more accessible to enzymatic hydrolysis. The microwave irradiation (MW) has special effect beyond the heat-generation, so we compared the efficacy of MW irradiation with the convective heat transfer, and both of thermal pretreatments were compared with the effect of alkaline treatments as well.

### Effect of different type of pre-treatment

The effects of different type of pre-treatments (PT) on the glucose yield are shown in the Figure 1 and Figure 2. Two different controls were used, for better understanding. At first there was used no enzyme and no PT as control1, than enzyme was added for the samples but any kind of PT were not applied as control2. The samples without enzymes have produced glucose in a slight extent under the repeatability limit of glucose assay method, meanwhile the enzyme treated EX and BY samples produced comparing amount of glucose c.

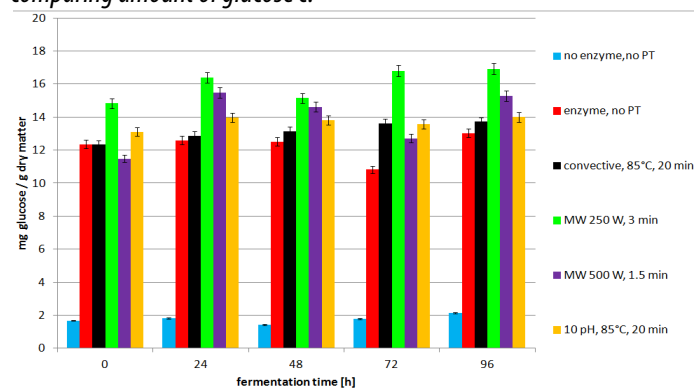


Figure 1. Effect of different pre-treatment on glucose production at EX samples

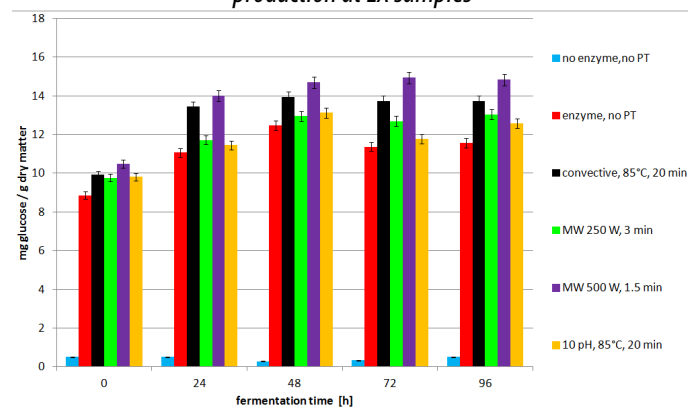


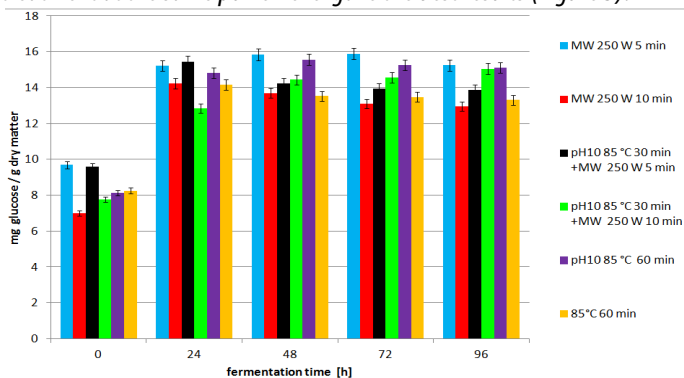
Figure 2. Effect of different pre-treatment on glucose production at BY samples

Considering the glucose yield the MW pre-treatment of EX at 250 W and 500W power level was more effective than conventional thermal pre-treatment. But the lower MW power resulted in lower glucose yield in the case of by-product tobacco than that of obtained from convective heated samples. The MW pre-treatment was the most effect both of samples, but the 500 W MW power treatment was the most successful at the BY, by contrast the 250 W MW power treatment was the best. Regarding the time difference: 500 W – 1.5 min, 250 W- 3 min, the irradiated energy was equal, but the effect of it, ie. the effect of the applied power level is different. This different could be explained by the different chemical composition and physicochemical structure of samples. The EX sample is the whole plant, it has lot of leaf as well, not only the stem, leaf stalk as the BY sample. The BY sample has a more complex structure, higher lignin content, lower surface area which resulted in lower ability for bioconversion. It is the reason of the absolute amount of glucose and the different at no enzyme no PT samples in the case of EX and BY.

The conventional heat treatment with the combination of the alkali treatment (pH10) affected also the glucose yield, but the increment was lower than obtained after MW treatments.

**Combined pretreatments**

Since the lower power level had better effect on sugar yield of EX samples, 250 W power level was applied at the combined pre-treatments with different time and combination with convective heat transfer (30 min) at pH 10. The shorter (5 min) MW pre-treatment had better glucose yield than longer (10 min) one but the previous applied 3 min long MW treatment at the same power level gave the best results (Figure 3).

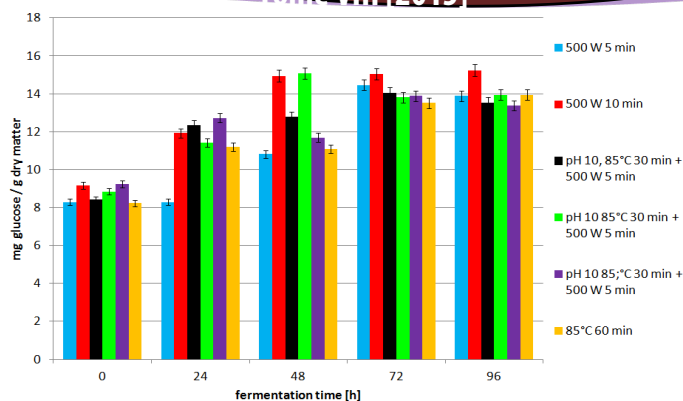


**Figure 3:** Combined pre-treatments effects on glucose yield at EX samples

The MW plus convective heat treated alkali samples had similar results as obtained from MW treatment; the MW treatment itself was the defining element at the efficiency, and the alkali pH had quite the same, very similar result. The convective heat transfer alone, even quite long time (60 min) has just the same effect on glucose yield than lower (250W) MW power with 10 min irradiation without alkaline addition.

The tendency was the same at BY samples (Figure 4). The main determinative treatment was the MW. Since these samples had much more thick cellulose fibres more dissipated energy was needed for exploration, i.e. 500 W 5 min was concluded as the most effective pre-treatment method.

Table 1 and Table 2 summarized the effect of combined pre-treatment on sugar yield in % and the maximum achieved sugar yield in mg/g<sub>dm</sub>.



**Figure 4.** Combined pre-treatments effects on glucose yield at BY samples

**Table 1.** Effectiveness of pre-treatments for BY samples

| pre-treatments                          | Max. sugar yield (mg/g <sub>dm</sub> ) | Effect of pre-treatment on sugar yield (%) | Time needed for max. sugar yield (h) |
|---|--|--|--------------------------------------|
| MW 500 W, 5 min                         | 16.523                                 | 57.302                                     | 72                                   |
| MW 500 W, 10 min                        | 17.402                                 | 60.069                                     | 96                                   |
| 10 pH, 85°C, 30 min +MW 250 W, 5 min    | 16.039                                 | 59.953                                     | 72                                   |
| 10 pH, 85°C, 30 min +MW 250 W, 10 min   | 17.207                                 | 58.726                                     | 48                                   |
| 10 pH, 85°C, 60 min                     | 15.868                                 | 66.435                                     | 72                                   |
| conductive heat treatment, 85°C, 60 min | 15.915                                 | 59.119                                     | 96                                   |

**Table 2.** Effectiveness of pre-treatments for EX samples

| pre-treatments                          | Max. sugar yield (mg/g <sub>DM</sub> ) | Effect of pre-treatment on sugar yield (%) | Time needed for max. sugar yield (h) |
|---|--|--|--------------------------------------|
| MW 250 W, 5 min                         | 15.89                                  | 60.938                                     | 72                                   |
| MW 250 W, 10 min                        | 14.24                                  | 50.934                                     | 24                                   |
| 10 pH, 85°C, 30 min +MW 250 W, 5 min    | 15.46                                  | 37.937                                     | 24                                   |
| 10 pH, 85°C, 30 min +MW 250 W, 10 min   | 15.05                                  | 48.458                                     | 96                                   |
| 10 pH, 85°C, 60 min                     | 15.55                                  | 47.723                                     | 48                                   |
| conductive heat treatment, 85°C, 60 min | 14.16                                  | 41.723                                     | 24                                   |

The data shows only MW treatment gives the bigger yield but the combined treatment enable to apply shorter fermentation time.

**SUMMARY**

The pre-treatment of two different type of tobacco samples was carried out in the experiments by using microwave irradiation. EX samples originated from the whole tobacco plants, it was a biomass plantation. The BY samples came from the tobacco factory, it was the byproduct of the tobacco production. At first step the effect MW pre-treatment on glucose yield was compare with the alkali and classical heat treated samples. The efficiency of different thermal and chemical pre-

treatments, their combination and the enzymatic hydrolysis was characterized by glucose yield measured by photometric glucose assay. The cellulose hydrolysis was carried out by cellulose and cellobiase enzymes. In MW experiments the power level (250 W and 500W) and irradiation time was varied.

Our experimental results show that in the case of BY samples the 500 W MW power, meanwhile the in the case of EX samples the 250 W MW power produced the maximum sugar yield. Increasing the duration of microwave irradiation, and applying combined alkaline/microwave process the pre-treatment with the magnetron power of 500W and 10 minutes resulted the maximum sugar yield from BY samples, but in combination with alkali treatment the fermentation period can be shortened, i.e. instead of 96 hour the maximum yield was achieved at 72 hours. In the case of EX samples the 250 W MW power and 5 minutes was the most effective, and the combination of MW and alkali treatment was proved suitable to reduce the overall time demand of hydrolysis process.

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