

ORIGINAL RESEARCH PAPER

**PRELIMINARY STUDY FOR OPTIMIZATION OF ENZYMATIC  
HYDROLYSIS OF WASTE CELLULOSIC MATERIALS**

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Lignocellulose is a generic term describing the main constituents in most plants, namely cellulose, hemicelluloses, and lignin. Cellulose is a glucose polysaccharide, hemicelluloses are polysaccharides with a backbone of different hexoses (glucose, mannose, galactose) and pentoses (xylan, arabinose), and lignin is a complex network of different phenyl propane units. The cellulosic materials are potential sources of ethanol. Steps of this process are saccharification of cellulose to reduce sugars, under enzymes action and to reduce sugars fermentation by yeast to obtain ethanol.

The aim of this study is to examine the influence of substrate concentration, temperature and pH upon enzymatic saccharification of waste cellulosic materials, based on office paper, newspaper and cardboard, in ratio of 1:1:1 (w/w) and reducing sugar accumulation dynamics in optimised conditions. The study has established optimal parameters: the ratio of enzyme:substrate as 0.5 EU/g substrate, temperature 48°C, pH 4.8 and addition of surfactant Tween 80 in proportion of 0.3 %, reported to the total volume of liquid. The reducing sugar yield was 35 mg reducing sugars/ g dry weight cellulosic waste.

**Keywords:** waste cellulosic materials, enzymatic hydrolysis, saccharification

### **Introduction**

Life is associated with waste production and the exploitation of cellulosic materials as renewable resources for bioproduct development could be a major challenge for biotechnology. Bioconversion processes have been developed for the utilization of renewable resources to produce useful chemicals and feed stocks (Bahrim, 2004). Production of ethanol (bioethanol) from biomass is a way to reduce both

consumption of crude oil and environmental pollution (Balat *et al.*, 2008). The importance of ethanol as a clean and safe transportation fuel has increased with the anticipated shortage of fossil fuel reserves and increased air pollution (Chen *et al.*, 2007). A directive was accepted that requests member states to establish legislation about utilization of fuels from renewable resources (Sun and Cheng, 2002). Polysaccharides in lignocellulosic materials including cellulose and hemicellulose can be hydrolyzed to monomeric sugars such as glucose and xylose, which can be further used for production of ethanol, xylitol, organic acid, and other chemicals. The hydrolysis of polysaccharides is usually catalyzed by hydrolytic enzymes (Chen *et al.*, 2007). The conversion of biomass to ethanol generally includes four steps: pre-treatment, hydrolysis of polysaccharides and oligosaccharides into monomer sugars, fermentation of sugars to ethanol and, finally, ethanol concentration to absolute alcohol (ethanol has to be concentrated to >99.8% to be used as motor fuel) (Cuevas *et al.*, 2010). Lignocellulosic ethanol possesses environmental and resource conservation advantages over both petroleum-based fuels and bioethanol, including potentially lower life cycle greenhouse gas (GHG) emissions and reduced usage of fossil fuel resources, particularly imported petroleum (Spatari *et al.*, 2010). High ethanol yield and low production cost need optimization of saccharification and fermentation processes (Kim *et al.*, 2008). Once the cellulose is converted into glucose, it can be easily fermented to produce ethanol (Champagne *et al.*, 2010).

The aim of this paper was to determine the possibility of improvement of enzymatic hydrolysis of a complex of three cellulosic wastes based on office paper, newspaper and cardboard in order to produce ethanol. For that purpose, the main objective was to determine the optimum saccharification parameters, such as substrate concentrations, temperature and pH which would result in better cellulose bioconversion of the waste cellulosic materials.

## Materials and methods

### *Substrate*

Lignocellulosic materials used in this study are waste cellulosic materials, a combination of office paper, newspaper and cardboard, in ratio of 1:1:1 (w/w). The used materials were pretreated as follows: milled at a vibratory ball milling, autoclaved of waste cellulosic materials at 120°C, in wet atmosphere, with H<sub>2</sub>SO<sub>4</sub>, 2% (w/w) concentration (1/7 w/v) for 24 hours, washed with distilled water until neutral pH and dried to a 98% dry matter. The hydrolysis essay procedure was performed in 250 ml flasks containing different concentrations of cellulosic material and cellulase (a commercial product), suspended in 50 ml final volume with a 50 mM citrate buffer, pH 4.8. The samples were incubated in a laboratory shaker, at 45°C and 150 rpm for 24 hours. The enzymatic reaction was stopped by essays keeping, for 10 minutes, in boiled water. The assays were performed in duplicate.

### *Enzyme*

The enzyme used in this study is Cellulase Onozuka R 10 (by *Trichoderma viridae*), 1 U/mg.

### *Saccharification conditions*

The substrate concentrations, temperature and pH values during saccharification process were varied as follows:

- *substrate concentrations* – a series of Erlenmeyer bottles containing different concentration, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 %, of pretreated cellulosic material and 1 mL enzyme, 0.1% (1UE/g) suspended in 50 ml final volume with a 50 mM citrate buffer, pH 4.8 and treated as it was described at 2.1.
- *temperature* – 0.2 g pretreated cellulosic material and 2 ml of enzyme, 0.1% (1UE/g) suspended in 50 ml final volume with a 50 mM citrate buffer, pH 4.8 and incubated at temperature values of: 10, 20, 30, 40, 50, 60 and 70°C, as it was described at 2.1.
- *pH* – the substrate consisting of 0.2 g pretreated cellulosic material and of 2 ml enzyme 0.1% (1UE/g) suspended in 50 ml final volume with distillate water and treated as it was described at 2.1. The pH values were adjusted with 0.01N H<sub>2</sub>SO<sub>4</sub> or 0.01N NaOH at values of: 2.0, 3.0, 4.0, 4.3, 4.5, 4.8, 5.6. The pH of samples was measured with a laboratory pH meter.

### *Hydrolysis yield determination*

Glucose concentrations were analyzed by using a dinitrosalicylic acid (DNS) assay. The reducing sugar reagent was the DNS reagent containing 1416 ml of deionized water, 10.6 g of dinitrosalicylic acid, 19.8 g of NaOH, 306 g of Rochelle salts (Na-K tartrate), 7.6 ml of phenol, and 8.3 g of sodium metabisulfite. A standard curve of glucose was used for the quantitative studies. The assays were performed in duplicate. The hydrolysis yield was expressed as mg reducing sugar/g dry weight cellulosic waste.

## **Results and discussion**

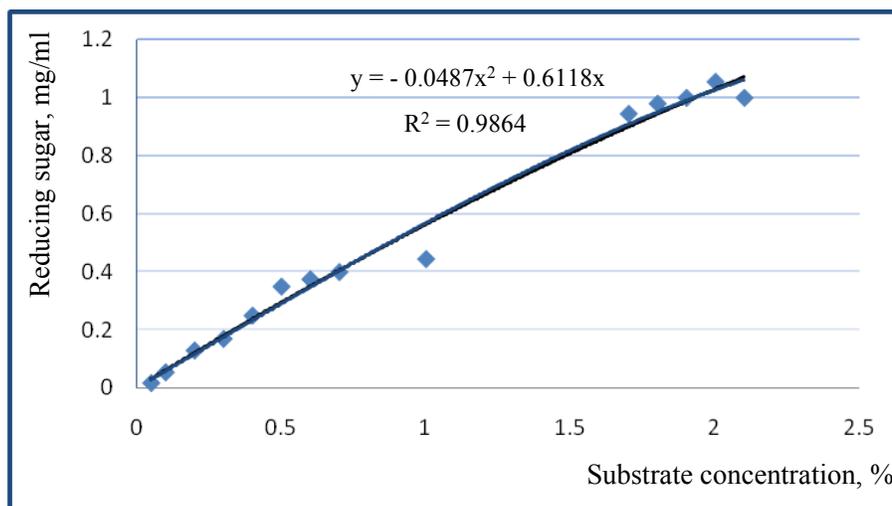
The influence of substrate concentration, temperature, pH, surfactants to biotechnological process and the dynamic accumulation of reducing sugars over enzymatic hydrolysis under optimal conditions were studied.

The influence of substrate concentration in bioconversion process is shown in Figure 1. It is noted that in waste cellulosic hydrolysis, the maximum amount of reducing sugars 1.05 mg/ml is obtained for a 2% substrate concentration, using 1 ml enzyme, in a concentration of 0.1% (1UE/g).

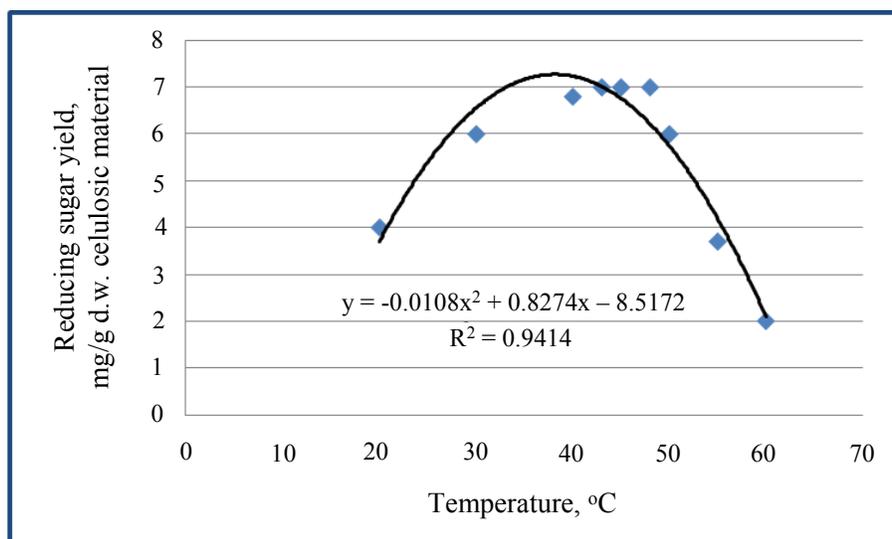
The smaller quantity of substrate hydrolyzed by the same amount of pure enzyme for cellulose pulp from waste can be explained by the fact that when using cellulosic waste (cardboard, paper, newspaper, office paper) enzyme can be inhibited by the presence of bleaching, printing ink, etc.

The influence of temperature in the bioconversion process is shown in Figure 2. Maximum enzymatic reaction yield is obtained at temperatures in the range 43-48°C respectively 7 mg reducing sugars/g dry weight cellulosic waste, temperatures higher or lower than these values do not determine the reducing of

sugar yield increases. This shows the optimum temperature range for the enzyme: cellulase Onozuka R10 of 43-48°C. Higher temperature causes enzyme inactivation, protein content due to distortion.



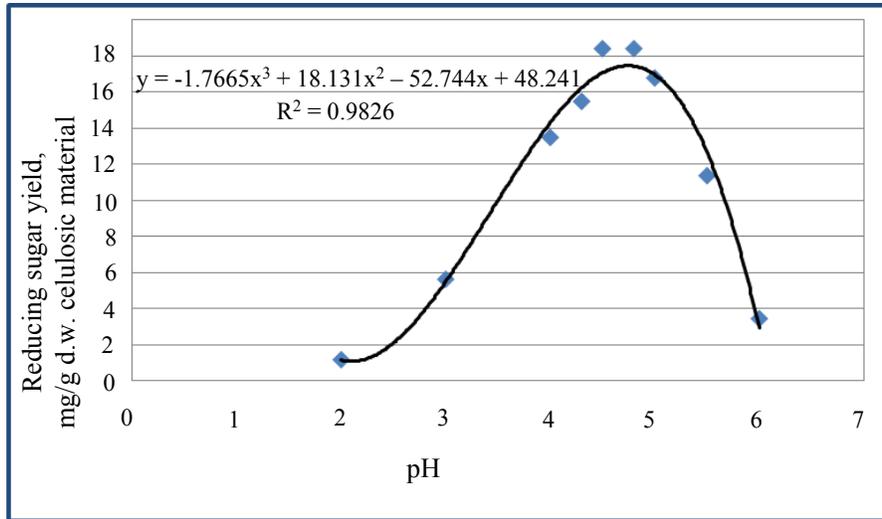
**Figure 1.** Reducing sugar production based on cellulose substrate concentration



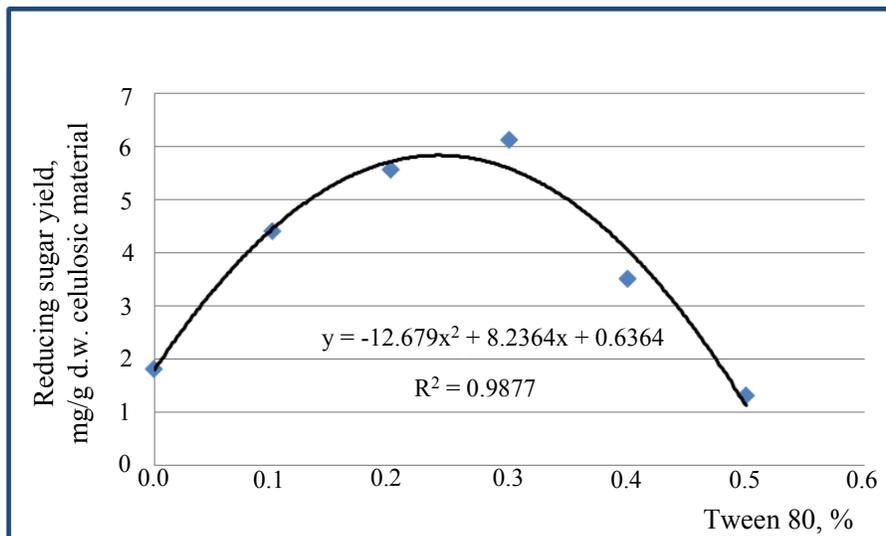
**Figure 2.** Reducing sugar yield variation based on bioconversion process temperatures

The influence of pH in bioconversion process was shown in Figure 3. The reducing sugar yield increases with pH increasing, with maximum value at: 4.5 to 4.8. It can be concluded that pH optimal for hydrolysis of cellulosic substrate is in a range of pH: 4.5-4.8.

To study the influence of added surfactants (Figure 4) on enzymatic hydrolysis of the cellulosic materials taken into the analysis was done as follows: 0.2 g sample prepared as above was treated with 2 ml of 0.1% enzyme, were added various concentrations of Tween 80, 0.1, 0.2, 0.3, 0.4 and 0.5%, the volume was brought to 50 ml with citrate buffer pH 4.8. Hydrolysis was performed at 45°C for 24 hours on a laboratory shaker, by stirring at 150 rpm.



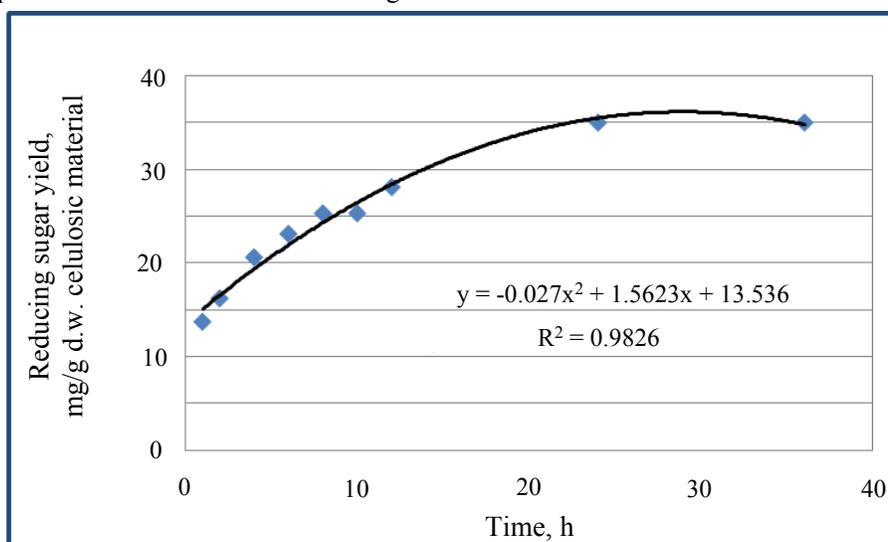
**Figure 3.** Reducing sugar yield variation based on pH variation during bioconversion process



**Figure 4.** Reducing sugar concentration release by enzymatic bioconversion in the presence of different Tween 80 concentrations

Enzymatic reaction is observed as calves increased by adding Tween 80, so reaction yield increases with increasing concentration in Tween 80 until the value of 0.3%, reaching 6.12 mg reducing sugars/g dry weight cellulosic waste. In conclusion the optimal concentration of Tween 80 on enzymatic hydrolysis of cellulosic waste is 0.3%.

For optimal substrate concentration determined by enzyme kinetics (corresponding to 2 g substrate/ 1UE) was tested the amount of reducing sugars accumulated after enzymatic hydrolysis at different time intervals: 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h. In Erlenmeyer flasks were placed 4g of substrate prepared as was described at 2.1, with 2 ml enzyme (1UE/ml) brought to final volume of 100 ml with citrate buffer, pH 4.8. The results are shown in Figure 5.



**Figure 5** Dynamic of reducing sugars accumulation during enzymatic hydrolysis of cellulosic waste substrate

It is noted that reducing sugar yield increases with increasing reaction time, reaching a value of 35 mg reducing sugars/g dry weight cellulosic waste.

### Conclusions

The optimal conditions for enzymatic bioconversion of a complex cellulosic waste material have been established as follows: the ration of enzyme: substrate 0.5 EU/g substrate, temperature 48°C, pH 4.8 and addition of surfactant Tween 80 in proportion of 0.3%.

In the optimized conditions were studied the dynamics of accumulation of reducing sugars over enzymatic hydrolysis of waste cellulosic materials. The reducing sugar

yield increased with increasing enzymatic hydrolysis time, so at 24h of reaction it was 35 mg reducing sugars/g dry weight cellulosic waste.

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