



Budapest University of Technology and Economics  
Department of Agricultural Chemical Technology

## **BIOFUELS FROM WASTES AND BY-PRODUCTS**

**PRODUCTION OF HYDROGEN AND ETHANOL BY FERMENTATION OF PAPER  
SLUDGE, CORN STOVER AND WOOD**

**PhD Thesis**

Zsófia Kádár

*Supervisor*  
*Dr Kati Réczey*

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# 1 INTRODUCTION AND SCOPE OF THE THESIS

The main problem of the XXI century is the increasing demand of fossil fuels meanwhile the supply store is limited and responsible for global environmental problems. The following two paragraphs summarize the background of the study.

Rising amount of greenhouse gases in the atmosphere are contributing to global warming. Some greenhouse gases occur naturally in the atmosphere causing a process called greenhouse effect. Without this natural phenomenon, the temperature would be so low, that life would not be possible on the Earth. Before the Industrial Revolution, human activity released very few gases into the atmosphere, but certain human activities (through population growth, fossil fuel burning, and deforestation) add to the levels of most of the naturally occurring gases thus changing the composition of Earth's atmosphere. Actions against the global warming are already exist (1992: Rio de Janeiro; 1997: Kyoto). The aim is to slow down global warming by reducing the emission of six primary greenhouse gases (carbon dioxide (as the major source from industry and automobiles), methane, nitrous oxide, hydrofluorocarbons, perfluorocarbons, and sulfur hexafluoride).

The deposit of oil is limited and would no longer be able to meet the continuously increasing demands (which is already 80 millions barrel daily) in the near future. The more oil will be pumped out worldwide, the more get used. According to latest calculations the demand could reach 10 millions barrel daily just in China. Oil is the most popular energy among the fossil fuels as it is cheap, safe and can be used for several applications. The oil crisis will begin when demand for oil consistently begins to exceed supply, not when the last drop of oil will be pumped. Of all the problems generated by fossil fuel use, the most challenging will be surviving the withdrawal from that use, after worldwide oil production peaks and begins to decline. Alternative possibilities are already exist (solar-, wind-, geothermal energy, biofuels) however applications of these possibilities are still objective of research. No one knows for sure when it will happen but the time is short....

The only solution for reducing CO<sub>2</sub> emission joined with increased energy demand is the utilisation of renewable fuels instead of fossil ones. Renewables are also promising options for the transportation sector in many countries, due to their potential to alleviate the crisis in the supply of oil derived liquid fuels while improving the urban air quality through lower emission. The 2003 biofuels directive (2003/30/EC) aims to increase the use of biofuels for transport, and in particular for road transport. The EU is aiming to replace 2% of all transport fossil fuels (petrol and diesel) with biofuels by 2005 and moreover 5.75% by 2010. However, as the share of raw material cost is calculated to be about 50% of total expenditures, the choice of feedstock is one of the most important factors affecting the economy of fuel alcohol production.

Hungary is poor in cheap, clean, high-quality domestic energy resources, more than 50% of energy consumption is satisfied by imports. However by-products from food- and paper industry, agriculture and forestry all provides various alternative energy sources thus in Hungary only biomass can currently be considered as a significant source of renewable energy.

Several forms of biomass resources exist (starch or sugar crops, oil plants, agricultural-, forestry- and municipal wastes) but of all biomass types the lignocellulosic feedstocks represent the most abundant and inexpensive global source of renewable resources.

Nowadays most of the fuel ethanol plants use sugar containing crops and grains (wheat and barley) as starch based raw material since today they are the only realistic alternative to produce ethanol. Developments are carried out all over the world on lignocellulosic materials.

The aim of the work was to examine industrial and agricultural wastes and by-products produced in Hungary for biofuel (hydrogen and ethanol) production. Among the possible production techniques used to make biofuel, biological fermentation methods were used in experiments for hydrogen and ethanol production. The experimental study mainly focuses on paper sludge, corn stover, spruce and willow as substrates. According to that, the thesis can be divided into three main parts:

1. Hydrogen fermentation on paper mill waste by (hyper)thermophilic microorganisms.
2. Ethanol production experiments on paper sludge by thermotolerant yeast.
3. Ethanol fermentation on steam pretreated corn stover, spruce and willow with an inhibitor resistant yeast strain.

## 2 MATERIALS AND METHODS

### RAW MATERIALS

Two industrial wastes, paper sludge and old corrugated cardboard (OCC), obtained from Dunapack Pulp and Paper Mill, Dunapack Paper and Packaging Ltd. (Budapest, Hungary) were used for the production of **hydrogen** and **ethanol** in SSF (Simultaneous Saccharification and Fermentation) experiments.

Paper sludge hydrolysate was used for **hydrogen** fermentation. Large scale hydrolysis was performed at a substrate concentration of 4% (w/v) in a pH and temperature controlled 31 L Braun fermentor by commercial enzyme preparations (Celluclast 1.5L and Novozyme 188). The pH and the temperature were set to 4.8 and 50°C, respectively.

Steam pretreated spruce willow and corn stover served as substrates for **ethanol** fermentations. Pretreatment of raw materials was carried out at Lund University (Sweden). Willow was pretreated under different conditions in the presence and in the absence of SO<sub>2</sub>.

### ENZYMES

For hydrolysis of paper sludge and for SSF fermentation experiments commercially available cellulase (Celluclast 1.5L) and β-glycosidase (Novozyme 188) preparations were applied (Novo Industri A/S, now Novozymes, Bagsvaerd, Denmark).

### MICROORGANISMS

For **hydrogen** fermentation *Thermotoga elfii* DSM 9442, *Thermotoga neapolitana* DSM 4359 and *Caldicellulosiruptor saccharolyticus* DSM 8903 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The cultures were grown in 100 ml sealed anaerobe serum flasks containing 30 ml of culture medium for 72h at 65°C, 48h at 80°C and for 24h at 70°C, respectively.

Commercial baker's yeast and *Kluyveromyces marxianus* Y01070 were obtained from the Budafok Yeast and Spirit Factory Ltd. (Budapest, Hungary) and from National Collection of Agricultural and Industrial Microorganisms, Szent István University – now Corvinus University (Budapest, Hungary) for SSF **ethanol** fermentation. The inoculum cultures were grown in 750 ml E-flasks containing 150 ml of culture medium at 30°C for one day.

*Saccharomyces cerevisiae* ATTC 26602 obtained from the American Type Culture Collection was used for **ethanol** production from steam pretreated lignocelluloses (spruce, willow and

corn stover). Starter cultures of *S. cerevisiae* ATCC 26602 were grown in 1000 ml cap flasks containing 500 ml of culture medium at 32°C for one day.

#### FERMENTATION ASSAYS

Small scale **hydrogen** fermentations were carried out in anaerobic 100-ml serum bottles with 30-ml volumes. The flasks were incubated under different temperatures: 65°C for *T. elfii*, 80°C for *T. neapolitana* and 70°C for *C. saccharolyticus*.

Batchwise anaerobic **hydrogen** production on paper sludge hydrolysate by *C. saccharolyticus* was performed in a 2-L bioreactor with initial 10% (v/v) inoculum volume. The temperature was controlled at 70°C and the pH was maintained at pH 6.4. *C. saccharolyticus* was cultivated with an agitation rate of 350 rpm. Hydrogen was continuously removed by sparging with nitrogen at a flow rate of 7 L/h.

SSF and NSSF (Nonisothermal Simultaneous Saccharification and Fermentation) **ethanol** fermentation experiments were performed in 750 ml E-flasks. Each flask contained 500 ml of culture with initial concentration of 6 wt.% DM of Solka Floc 200, OCC or paper sludge. In all cases, the culture medium was supplemented with cellulase enzymes (Celluclast 1.5 Novozym 188). The living cell content in the medium was  $2 \times 10^9$  cells/ml after inoculation. The flasks were incubated in a rotary shaker at 40°C for 96 h. The pH of the fermentation broth was set to 4.4 - 5.3. In case of NSSF experiments, the same procedure was followed except a 24 h prehydrolysis at 50°C, was followed by inoculation with yeast cells and incubation at 30°C.

Batch **ethanol** fermentations on the hemicellulose hydrolysates of steam pretreated lignocelluloses with additional glucose (according to cellulose content) were carried out in stirred flasks. Experiments were performed in 0.5 l capped flasks contained 100 ml of fermentation broth agitated at 300 rpm and incubated in waterbath at 32°C until the end of the fermentation. The pH of the broth was adjusted to 4.0 to avoid bacterial contamination.

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#### ANALYTICAL ASSAYS

At **hydrogen** fermentations changes in biomass concentration throughout the fermentation process were followed by optical density (OD) measurement at 580 nm. Quantitative biomass concentration was assayed applying microbiuret cell protein determination. Glucose, xylose, and organic acids were analyzed by high-performance liquid chromatography (HPLC). Production of hydrogen and CO<sub>2</sub> was determined by gas chromatography.

Consumed sugars and products of **ethanol** fermentation were analyzed on HPLC. On the supernatants of steam pretreated lignocellulosics, changes in biomass concentration throughout the fermentation process were measured by optical density measurement at 700 nm. Production of carbon dioxide was monitored on-line using a HaloteC BAM-6 module.

### 3 NEW SCIENTIFIC RESULTS

#### RESULTS ON HYDROGEN FERMENTATION

1. All the tested (hyper and extreme)thermophilic microorganisms *Thermotoga elfii*, *Thermotoga neapolitana*, and *Caldicellulosiruptor saccharolyticus* can grow and produce hydrogen on paper sludge hydrolysate. The effect of additional medium components (yeast extract, trace elements and salts) required for these microorganisms for growth and optimal hydrogen production on paper sludge hydrolysate were studied in experimental series based on 2<sup>3</sup> factorial design in small serum bottles.

During fermentations *T. elfii* produced a high amount of hydrogen, but needed yeast extract to do so. As it is a halophilic bacterium, 1% NaCl is also required.

*T. neapolitana* also produced high amount of hydrogen from paper sludge hydrolysate, but only in the presence of salts in the medium.

*C. saccharolyticus* seemed to be less dependent on additional medium components, as the hydrogen production on paper sludge hydrolysate was not stimulated by the addition of yeast extract, salts or trace elements.

2. *C. saccharolyticus* was able to grow directly on paper sludge hydrolysate on a larger scale under controlled conditions. The obtained 51% yield on paper sludge hydrolysate was 84% of the yield achieved on control medium (glucose and xylose). Hydrogen production on lean medium was comparable to fermentations on complete medium showing that the requirements for other salts and trace elements are low.

#### **RESULTS ON ETHANOL FERMENTATION**

1. Commercially available baker's yeast and the thermotolerant *Kluyveromyces marxianus* were able to produce ethanol on paper sludge in simultaneous saccharification and fermentation (SSF) process. There were no significant differences observed between the two strains at 40 °C, with a yield of 0.31–0.34 g ethanol/g cellulose. SSF with temperature profiling (NSSF) was not resulted in higher ethanol yields.
2. *Saccharomyces cerevisiae* ATCC 26602 was able to take part in ethanol fermentation at low initial pH (pH4), which is necessary to avoid bacterial contamination.
3. Steam pretreated willow, spruce and corn stover, after proper pretreatment, seem to be a possible substrate for economically profitable industrial ethanol production with final ethanol concentration of 5vol%. Impregnation with SO<sub>2</sub> did not affect the applied microorganism, but was found to be necessary to improve the recovery of hemicellulose.
4. The inhibitor tolerance of the selected *Saccharomyces cerevisiae* ATCC 26602 could be improved with continuous adaptation on steam pretreated spruce matrix. Under the same circumstances and inhibitor concentrations, the adapted yeast was able to ferment 5vol% ethanol, while the original, non-adapted, yeast strain was unable for ethanol fermentation at all.

## **4 LIST OF PUBLICATIONS**

This thesis is based on the following papers:

- Kádár, Zs., de Vrije, T., Budde, M., Szengyel, Zs., Réczey, K. and Claassen, P. A. M. (2003) Hydrogen production from paper sludge hydrolysate. *Appl. Biochem. Biotech.* **105**, 557-566.
- Kádár, Zs., de Vrije, T., van Noorden, G. E., Budde, M. A. W., Szengyel, Zs., Réczey, K. and Claassen, P. A. M. (2004) Yields from glucose, xylose and paper sludge hydrolysate during hydrogen production by the extreme thermophile *Caldicellulosiruptor saccharolyticus*. *Appl. Biochem. Biotech.* **113-116**, 497-508.

- Kádár, Zs., Szengyel, Zs. and Réczey, K. (2004) Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Ind. Crops Prod.* **20**, 103-110.
- Kádár, Zs., Maltha, S. F., Szengyel, Zs., Réczey, K. and de Laat, W. Ethanol fermentation on various pretreated and hydrolyzed substrates at low pH. Manuscript.

Other related papers by the same author:

- Varga, E., Kádár, Zs., Schuster, K. C., Gapes, J. R., Szengyel, Zs. and Réczey, K. (2002) Possible substrates for acetone–butanol and ethanol fermentation based on organic by-products. *Hung. J. Ind. Chem.* **30**, 19-27.
- Kádár, Zs., Varga, E. and Réczey, K. (2001) New substrates of biofuel. *Magyar Mezőgazdaság*. V 56. N. 19, 32-33. (in Hungarian)
- Claassen, P. A. M., Budde, M. A., van der Wal, F. J., Kádár, Zs., van Noorden, G. E. and de Vrije, T. (2002) Biological hydrogen production from biomass by thermophilic bacteria. *Proceedings of the 12th European Conference on Biomass for Energy, Industry and Climate Protection, June 17-21, Amsterdam, The Netherlands*, Volume I. 529-532.
- Kádár, Zs., de Vrije, T., Szengyel, Zs., Claassen, P. A. M. and Réczey, K. (2003) Hydrogen as alternative fuel. *Proceeding of the Technical Chemical Days'03, April 8-10, Veszprém, Hungary*, 372-377. (in Hungarian)
- Kádár, Zs., Kis, B. and Réczey, K. (2000) Biofuel production from paper sludge. *Proceeding of the Technical Chemical Days', April 25-27, Veszprém, Hungary*, 244-248. (in Hungarian)

Oral and poster presentations related to the thesis:

- Kádár, Zs., Balogh, Cs., Maltha, S. F., Szengyel, Zs., Réczey, K. and de Laat, W. (2005) Effect of inhibitors on pretreated lignocellulosic biomass on the ethanol yield. 14<sup>th</sup> European Biomass Conference and Exhibition: Biomass for Energy, Industry and Climate Protection. October 17-21, Paris, France.
- Kádár, Zs., Balogh, Cs., Maltha, S. F., Szengyel, Zs., Réczey, K. and de Laat, W. (2005) Yeast adaptation on pretreated lignocellulosic substrates. Renewables Resources and Biorefineries. September 19-21, Ghent, Belgium.
- Kádár, Zs., Maltha, S. F., Szengyel, Zs., Réczey, K., de Laat, W. (2004) Ethanol fermentation of various pretreated and hydrolyzed substrates. 26<sup>th</sup> Symposium on Biotechnology for Fuels and Chemicals, May 9-12, Chattanooga, TN, USA.
- Kádár, Zs., de Vrije, T., van Noorden, G. E., Budde, M. A. W., Szengyel, Zs., Réczey, K., Claassen, P. A. M. (2003) Anaerobic fermentation of paper sludge hydrolysate to hydrogen by extreme thermophilic bacteria. 25<sup>th</sup> Symposium on Biotechnology for Fuels and Chemicals, May 4-7, Colorado, USA.
- Kádár, Zs., de Vrije, T., Szengyel, Zs., Claassen, P. A. M., Réczey, K. (2003) Hydrogen as alternative fuel. Technical Chemical Days'03, April 8-10, Veszprém, Hungary.
- Kádár, Zs., de Vrije, T., Szengyel, Zs., Réczey, K., Claassen, P. A. M. (2002) Hydrogen production from paper sludge hydrolysate. 24<sup>th</sup> Symposium on Biotechnology for Fuels and Chemicals, April 28-May 1, Gatlinburg, TN, USA.
- Claassen, P. A. M., de Vrije, T., de Haas, G., Budde, M. A., Kádár, Zs., van der Wal, F. J., van Noorden, G. E. (2002) Utilisation of biomass for dark hydrogen fermentation. Biohydrogen 2002, April 21-24, Ede, The Netherlands.

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- Kádár, Zs., Szengyel, Zs., Réczey, K. (2002) Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. International Congress & Trade Show Green-Tech 2002 with the European Symposium Industrial Crops and Products, April 24-26, The World Horticultural Exhibition Floriade, The Netherlands.
- Kádár, Zs., Kis, B., Szengyel, Zs., Réczey, K. (2001) Simultaneous saccharification and fermentation experiments on various substrates. 23<sup>rd</sup> Symposium on Biotechnology for Fuels and Chemicals, May 4-9, Colorado, USA
- Kádár, Zs., Réczey, K. (2001) Bioethanol from wastes. Technical Chemical Days'01, April 24-26, Veszprém, Hungary.
- Kádár, Zs., Réczey, K. (2001) Wastes, as feedstocks for bioethanol production. "BUTE Industrial Open Day", Bioenergy, Biofuels Symposium, February 28, Budapest, Hungary.
- Kádár, Zs., Réczey, K. (2001) Ethanol production by simultaneous saccharification and fermentation. 301. Scientific Conference, February 23, Budapest, Hungary.
- Kádár, Zs., Kis, B., Réczey, K. (2000) SSF on cellulose based substrates. "Lippay János – Vas Károly" Scientific Conference, November 6-7, Budapest, Hungary.
- Kádár, Zs., Kis, B., Réczey, K. (2000) Biofuel from paper sludge. "Chemist Days", April 25-27, Veszprém, Hungary.
- Kádár, Zs., Réczey, K. (2000) Bioethanol from industrial wastes. Chemist Conference, November 26-28, Kolozsvár, Romania
- Kádár, Zs., Varga, E., Réczey, K. (1999) Possible substrates for ethanol fermentation. 3<sup>rd</sup> European Motor Biofuels Forum, October 10-13, Brussels, Belgium.
- Varga, E., Kádár, Zs., Réczey, K. (1999) Possible substrates for ABE fermentation. Conference on the Applied Acetone Butanol Fermentation. September 16-18, Krems, Austria.