NARDI FUNDULEA, ROMANIA
www.incda-fundulea.ro

ROMANIAN AGRICULTURAL RESEARCH, NO.27, 2010
Print ISSN 1222-4227; Online ISSN 2067-5720

UTILIZATION OF LAST GENERATION ENZYMES FOR INDUSTRIAL USE IN ORDER TO OBTAIN BIOETHANOL FROM LOCALLY AVAILABLE AGRICULTURAL RENEWABLE RESOURCES

Mihaela Begea[1], Mariana Vlădescu[1], Gheorghe Bâldea[1], Cornelia Cîmpeanu[1]
Cristina Stoicescu[1], Marius Toboşaru[2], Paul Begea[1]

[1] ICA R&D, Splaiul Independentei 202, Bucharest, District 6, Romania. E-mail: ela_begea@yahoo.com
[2] EDR Ingredients, Conacul Cantacuzino Paşcanu, 617140 Costişa, Neamț County. Romania
E-mail: marius_tobosaru@enzymes.ro

ABSTRACT

The paper presents the results of laboratory and pilot studies to produce bioethanol from starchy raw-materials (namely maize) using a very high gravity system (VHG). The aim of the experiments was the testing of the last generation of enzymes for industrial use produced by companies: NOVOZYMES and DANISCO (GENENCOR enzymes) and the establishment of most suitable pilot technology using these enzymes. The evolution of mash parameters during liquefaction, saccharification and fermentation was monitored during pilot experiments and finally the yield in ethanol and specific consumptions were calculated, in order to estimate the process effectiveness.

Key words: first generation bioethanol, enzymatic degradation of starch, enzymes for industrial use, maize, agricultural renewable energy sources (RES), very high gravity technology (VHG).

INTRODUCTION

Biofuels are fuels derived from renewable biomass in order to be used for the combustion engines or for other forms of energy generation. They replace partially or totally the fossil fuels.

The main liquid biofuel are bioethanol, produced through the fermentation of sugars and starch derived from selected agricultural resources, and biodiesel, produced through the transesterification of vegetal oils (Flammini, 2008).

The production of ethanol from energy crops traditionally cultivated for food is limited by the geographical factors and agriculture. Bioethanol derived from cereals or sugar cane was proved to be feasible at industrial level (Mousdale, 2008).

The enzymatic conversion of starch to glucose is performed using mixtures of enzymes acting in different stages of hydrolysis of starch molecule. For liquefaction an alpha amylase is used, in order to break down starch into maltodextrins and rapidly reduce the viscosity of the mash, and for saccharification a glucoamylase is used. Maintaining an optimum glucose concentration during fermentation requires a high-activity glucoamylase with consistent, superior quality (NOVOZYMES).

As starch source the cereals are the main resources, especially in the case of Romania (Stroia et. al., 2007a,b). Because of the different composition of starch derived from various botanical sources, the liquefaction and saccharification steps have specific pathways for every substrate. In addition, every enzyme obtained from different strains of micro organisms acts differently on these substrates.

Up to now, the normal mash concentration in dry matter in ethanol industry was between 14-20%, depending on the ratio ground maize/water. The values for mash concentrations generally recommended by industrial enzymes producers are 16-18% d.m. These values of dry matter content can ensure the minimum ethanol concentration of 8% v/v and thus the profitability of distillation and refinery installations.

In the case of modern bioethanol technologies, using last generation industrial

Received August 26, 2009; accepted January 14, 2010

enzymes, addressing exclusively bioethanol production, are used and thus the work with concentrated mashes (Very High Gravity system) can be applied. In this case the concentration of maize mashes can be up to 36% d.m.

The optimal bioconversion of sugar to ethanol demands a yeast strain able to tolerate high ethanol concentrations, because the ethanol inhibits the multiplication and fermentation processes (Roehr, 2001).

Most of starchy raw materials used for bioethanol production provide all nutrients beside carbon, necessary for the bioconversion. The addition of nutrients is necessary in particular cases and based on the experience with VHG system the classical fermentation media are enriched in free amino acids and peptides during the initial growing stage.

Another technological alternative is to use some special enzymes – industrial proteases – that can release into the mash amino acids and peptides. This release plays a major role in increasing of cells density and reducing the fermentation duration (The Alcohol Textbook, 2003; Mousdale, 2008). This aspect was neglected till now in Romanian ethanol industry and during the pilot experiments we tested the supply with assimilable nitrogen in cereals (maize) mashes.

For the mashes obtained in VHG system the fermentative capacity of yeast is greater in the case of the addition of urea in comparison with simple mashes and the fermentation is improved (Pham et Wright, 2008; Thomas et al., 1993).

MATERIAL AND METHODS

The pilot experiments using maize as locally available renewable energy resource had the following main goals:

- testing of last generation of enzymes for industrial use produced by two renowned companies: NOVOZYMES and DANISCO (GENENCOR enzymes);
- establishing the most suitable pilot technology using these industrial enzymes.

Maize mashes with high gravity having a concentration in soluble dry matter of ca. 35 g/100 ml mash were obtained using a ration water: ground maize = 2 : 1. There were no troubles related to the viscosity of the mash, due to the specific characteristics of maize.

The addition of 1000 ppm urea was performed to provide assimilable nitrogen at fermentation.

The pilot installation included fermentation vessels with total volume of 20 liters, 100 liters and 140 liters, endowed with monitoring and control devices of temperature, pH and dosage (raw material, nutritive salt solutions).

Dried yeast with a high ethanol tolerance for the production of industrial ethanol from grains such as maize, barley, wheat etc. with a final ethanol concentration of up to 18% (v/v) was used. This strain displays a higher yield in ethanol than standard yeast, even at high temperatures (up to 40°C) and the strain is suitable for VHG processes (up to 36% dry matter) and Simultaneous Saccharification and Fermentation (SSF) processes. The yeast is produced under the brand Ethanol Red by the company FERMENTIS (France).

Two series of experiments, one for every category of enzymes were performed.

During the first series of experiments, the maize starch was hydrolyzed using industrial enzymes produced by the Danish company NOVOZYMES.

The following enzymes were used:

- Liquozyme SC DS for liquefaction of starch. It breaks down starch into maltodextrins and rapidly reduces the viscosity of the mash;
- Spirizyme Fuel for saccharification.

In the case of maize mashes the addition of calcium ions becomes necessary because the existing phytic acid blocks the calcium uptake during the mashing process. This was the reason for the addition of calcium in form of calcium chloride at starch hydrolysis.

The higher is the liquefaction temperature the more intense is the calcium blocking process. In this respect we performed experiments with lower liquefaction temperature (83-86°C), although the classical recommended liquefaction temperature for maize starch is 90°C. This could represent a significant innovation for the bioethanol plants in terms of energy savings and cooling water (mash heating till 90°C, further cooling till the

saccharification temperature, respectively the pitching temperature for yeast).

The maize used for this series of experiments had the following quality parameters:

- dry matter content: 11.64%;
- starch content: 65.25% d.m.

The optimized technological diagram used for the first series of experiments was the following (Stroia et. al., 2007a,b; Begea et. al., 2009):

- maize milling using a hammer mill with a 1.5 mm sieve;
- ratio maize ground : water = 1 : 2 (1 kg ground maize : 2 liters water);
- addition of Liquozyme SC DS in dose 0.2 g/kg maize ground;
- addition of 2 ppm Ca^{+2} (as calcium chloride);
- mashing 10 minutes at 50°C;
- raising of temperature of mash till 85°C;
- liquefaction rest at 85°C for 90 minutes;
- cooling of mash at 70°C;
- addition of Spirizyme Fuel in dose 0.5 ml/kg maize;
- cooling of mash till the pitching temperature of 32°C;
- pitching with Ethanol Red yeast as a 20% dry solids solution in dose of 0.5% w/v to the mash;
- alcoholic fermentation at following parameters:
  - temperature of 32°C;
  - pH 4,5 – 4,8;
  - addition of urea in dose of 1000 ppm;
- fermentation was performed at pH 4.5 through H_2SO_4 1/6 addition and the maintenance at this value during test performance through addition of solution 25% NH_3 (Novozymes, 2008; Stroia et. al., 2007b).

The second series of pilot experiments, the maize starch was hydrolyzed using industrial enzymes provided by DANISCO Company (GENENCOR enzymes).

The technology applied in the case of GENENCOR enzymes was different from the NOVOZYMES technology, the main difference being that the liquefaction, saccharification and fermentation were performed simultaneously and for liquefaction and saccharification an enzymes complex containing both enzymes (alpha amylase and glucoamylase) was used.

The working parameters were different in comparison with NOVOZYMES technology. During hydrolysis of starch the glucose is continuously released and directly fermented by the yeast.

The GENENCOR enzymes used in pilot experiments were Stargen 001 and Fermgen.

Stargen 001 is a granular starch hydrolyzing enzyme containing *Aspergillus kawachi* alpha amylase expressed in *Trichoderma reesei* and a glucoamylse from *Aspergillus niger* that work synergistically to hydrolyze granular starch substrate to glucose.

Fermgen is an acid proteolytic enzyme characterized by its ability to hydrolyze proteins under low pH conditions. The fungal protease is obtained by controlled fermentation of a genetically modified selected strain of *Trichoderma reesei (*GENENCOR, 2008).

The optimized technological diagram used for the second series of experiments using GENENCOR enzymes was the following (Stroia et al., 2007b):

- maize milling using a hammer mill with a 0.6 mm sieve (min. 95% grind-size of 0.59 mm);
- ratio maize ground : water = 1 : 2 (1 kg ground maize : 2 liters water);
- dosage of ground maize in water and stirring 1 hour at the initiation of process (30°C for charges 1÷3 and 35°C for charges 4÷6). Further the process was performed under still and continuous stirring, allowing the permanent maintenance of ground maize in suspension;
- addition of enzymes and urea after 1 hour in the following manner:
  - dosage of STARGEN in dose of 1.7 g/kg maize;
  - dosage of FERMGEN in dose of 0.4 g/kg maize;
  - dosage of urea in dose of 1000 ppm.

- pH adjustment at 4.2 using H_2SO_4 1/6;
- pitching with Ethanol Red yeast as a 20% dry solids solution in dose of 0.5% w/v to the mash.

The maize used for this series of experiments had the following quality parameters:

- dry matter content: 10.21%;
- starch content: 64.31% d.m.

Six pilot experimental charges, three at process temperature of 30°C (charges 1÷3) and three at process temperature of 35°C (charges 4÷6) were performed.

During pilot experiments using maize as raw material the evolution of ethanol content, total residual sugar and glucose content in mash were traced every 2 hours.

RESULTS AND DISCUSSION

The procedure using high concentrated mashes (VHG = Very High Gravity) has the following technological and economical advantages:

- the water consumption is appreciably reduced;
- the capacity of the factory is increased and the volume of fermentation vessels is more effectively used;
- the work productivity is improved;
- the incidence of microbiological contamination is reduced;
- the energy necessary for the distillation decreases because the fermented mash has a higher content in ethanol (16-23% v/v);
- the resulting spent-grains can be used as a valuable by-product.

Figure 1 presents the evolution of ethanol content, total residual sugar and glucose content in mashes liquefied and saccharified with Novozymes industrial enzymes using the specific optimized diagram. The results represent the average value for 10 pilot experimental charges.

The following observations can be drawn analyzing the figure 1:

- the ethanol content increased from 0 to 15.41% v/v;
- the glucose content decreased from 8.5% at the beginning of fermentation till 0.23% at the end of fermentation;
- the total residual sugar content decreased from 23.92% at the beginning of fermentation till 1.21% at the end of fermentation.

Practical yield in ethanol was 93.90% from the theoretical yield, this value representting an excellent yield. The calculated maize specific consumption was 259.57 kg maize/100 liters absolute ethanol.

The results registered for the pilot experiments of maize mashes processed in order to obtain bioethanol using GENENCOR industrial enzymes are presented in table 1.

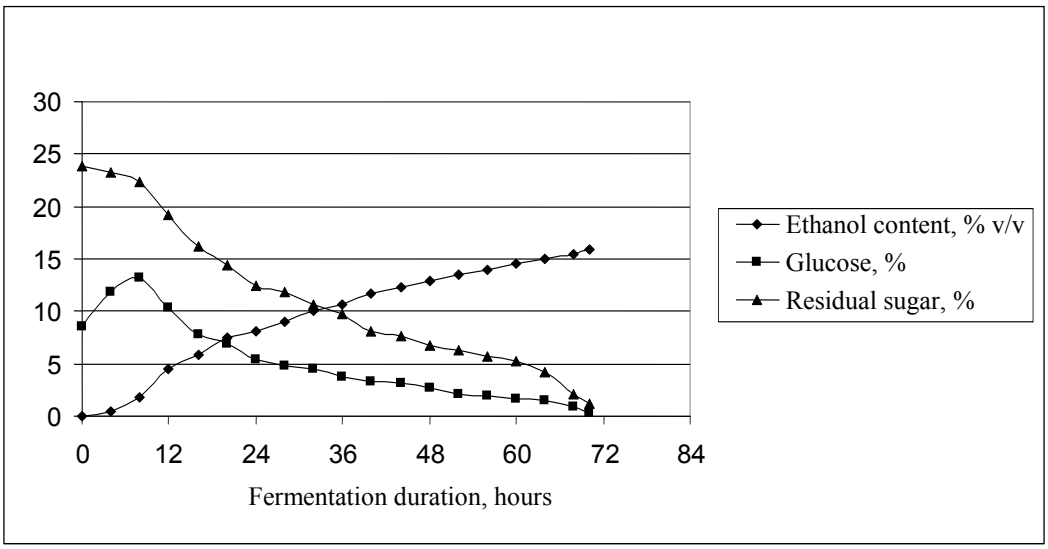

Figure 1. Evolution of ethanol content, total residual sugar and glucose content in mashes liquefied and saccharified with Novozymes industrial enzymes

The following observations can be drawn analyzing the table 1:

- the results obtained for the pilot experiments performed at 30°C were lower than these performed at 35°C, justified by the ethanol content and mainly by the residual sugar at the end of fermentation;
- fermentation process in the case of charges 1÷3 stopped after more than 72 hours, the normal and recommended value for the end of fermentation;
- the optimal temperature for the GENENCOR enzymes was 35°C.

Table 1. Ethanol content, total residual sugar and glucose content in mashes liquefied and saccharified with GENENCOR industrial enzymes at the end of fermentation process

Charge	Ethanol content % v/v	Residual sugar %	Glucose %	Duration of fermentation process hours
Charge no. 1	11.57	7.41	2.27	79
Charge no. 2	13.56	6.13	1.32	77
Charge no. 3	13.71	6.09	1.25	73
Charge no. 4	14.89	1.18	0.20	69
Charge no. 5	15.18	1.21	0.23	70
Charge no. 6	14.82	1.20	0.21	71

Practical yield in ethanol was 90.55% from the theoretical yield, the value representing a very good yield. The calculated maize specific consumption was 267.38 kg maize/100 liters absolute ethanol.

CONCLUSIONS

All pilot fermentation experiments were performed in the range of 60÷80 hours, the addition of urea leading to a reduction of fermentation duration up to 18%.

For a specific volume of fermentation vessel the maize ground weights used in VHG system were higher with up to 31% in comparison with classical fermentation system, using normal mash gravity. The increase in ethanol concentration in fermented mashes was in the range of 35÷56%. Decreasing water content used for mash preparation had a significant contribution to the economy saving for heating and cooling of maize mashes, and also for the distillation in order to obtain bioethanol.

In the case of NOVOZYMES products and for the proposed optimized pilot technology, the liquefaction temperature of 85°C was sufficient in order to ensure the process of obtaining bioethanol with very good yields and specific consumption.

The evolution of glucose concentration and total sugar in fermented maize mash, that lead to a good yield in ethanol and low residual sugar content at the end of process, is first of all, a result of the performance of the industrial enzymes. This good performance to led obtaining a suitable fermentable substrate, that allowed reaching high ethanol concentration and very good ethanol yields.

The optimal temperature for the simultaneous process of liquefaction, saccharification and fermentation in the case of GENENCOR industrial enzymes was 35°C.

Diagrams proposed to be applied for maize processing into bioethanol, using last generation industrial enzymes specially created for bioethanol, ensure very good yield in ethanol and specific consumption of raw materials.

Acknowledgements

This paper presents the results of the project IDEI 321/2007 „*Researches for establishing processing biotechnologies to use renewable indigenous agricultural resources*”, supported by the Romanian National Plan for Research – Development and Innovation of the Ministry for Education and Research.

REFERENCES

Begea, M., Vlădescu, M., Bâldea, G., Cîmpeanu, C., Stoicescu, C., Begea, P., 2009. *Isolation and selection of high ethanol producing yeast strains.* Journal of Agroalimentary Processes and Technologies, 15, 1: 107-111.

Flammini, A., 2008. *Biofuels and the underlying causes of high food prices*. FAO, Roma: 1-31.

Mousdale, D., 2008. *Biofuels: Biotechnology, Chemistry, and Sustainable Development*. CRC Press, New York: 96-102.

Pham, T.K., Wright, P.C., 2008. *The proteomic response of Saccharomyces cerevisiae in very high glucose conditions with amino acid supplementation.* Journal of Proteome Research, 7, 11: 4766-4774.

Roehr, M., 2001. *The biotechnology of ethano.:* Classical and Future Applications. Edited by M. Roehr, Weinheim, Cambridge University Press, Wiley-VCH, 90.

Stroia, I., Begea, M., Begea, P., 2007a. *From the agricultural raw materials as energy crops to bioethanol*. Proceedings of the 1st International Bioenergy Forum, Sofia, Bulgaria: 67-69.

Stroia, I., Begea, M., Begea, P., Vlădescu, M., 2007b. *Utilisation of industrial enzymes to produce bioethanol from autochthonous energy crops*. Journal of Agroalimentary Processes and Technologies, Volume XIII, No.2: 263-270.

Thomas, K.C., Hynes, S.H., Jones, A.M., Ingledew, W.H., 1993. *Production of fuel alcohol from wheat by VHG technology*. *Effect of sugar concentration and fermentation temperature.* Applied Biochemistry and Biotechnology, 43, 3: 211-226.

*** 2008, GENENCOR technical prospects,

*** 2008, NOVOZYMES technical prospects,

*** 2003, The Alcohol Textbook, 4th Edition, Nottingham University Press: 113-115.