CHEMICAL ENGINEERING TRANSACTIONS
VOL. 50, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Katharina Kohse-Höinghaus, Eliseo Ranzi

**ISBN** 978-88-95608-41-9; **ISSN** 2283-9216
DOI: 10.3303/CET1650028

# Ethanol Fermentation of Cellulosic Hydrolysate from Sugarcane Bagasse to Develop Stand-Alone Second Generation Ethanol Plant

Celina K. Yamakawa*[a,d], Sebastian T. Rojas[b], Victor C. Geraldo[c], Elmer A. C. Rivera[a], William E. Herrera[d], Antonio Bonomi[a,d], Carlos E. V. Rossell[a,d], Rubens M. Filho[a,c,d]

[a] Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Caixa Postal 6192, CEP 13083-970, Campinas, SP, Brazil.
[b]School of Food Engineering, State University of Campinas (UNICAMP), Campinas, SP, Brazil.
[c]Integrated Post-Graduated Program in Bioenergy, Campinas, SP, Brazil.
[d]School of Chemical Engineering, State University of Campinas (UNICAMP), Campinas, SP, Brazil.
celina.yamakawa@bioetanol.org.br

In this study, the ethanol fermentation of cellulosic hydrolysate produced from hydrothermal pretreatment of sugarcane bagasse, followed by detoxification and enzymatic hydrolysis was investigated. It was conducted on fermentability, and nutrient supplementation requirement. Fermentability was evaluated through ethanol fermentation with cell recycle for eight sequencing batches. In the first cycle, glucose was converted mostly to ethanol and cells without nutrients addition. In the following batches, nitrogen and phosphate supplementation to achieve a successful fermentation was required. The kinetics of cell growth, substrate consumption and ethanol production during fermentation were described using a mechanistic model. Specific ethanol productivity of 1.34 kg/m$^3$.h, yield of 90 % and cell viability of 93 % were obtained. Nutrient supplementation was assessed using a central composite design to investigate the effect of molasses, urea and diammonium phosphate (DAP) addition on productivity. Statistical analysis showed that only the effect of molasses addition was significant on productivity at 95% confident level.

## 1. Introduction

Ethanol production from lignocellulosic biomass named as Second Generation (E2G) includes pretreatment, enzymatic hydrolysis of cellulignin, ethanol fermentation of hexose and pentose, and ethanol recovery. Bagasse and straw are the main lignocellulosic material from sugarcane, composed by cellulose, hemicellulose, lignin, ash, and extractives. The application of an appropriate process to breaking down the hemicellulose and cellulose fractions results into liquid streams of hemicellulosic hydrolysate called C5 liquor, and into cellulosic hydrolysate called C6 liquor. It is necessary to remove inhibitors compounds generated from the degradation of sugars and lignin during pretreatment when hemicellulosic hydrolysate is converted into ethanol or other bioproducts through fermentation (Canilha et al. 2012). The cellulosic hydrolysate stream could be blended with the substrate stream of conventional ethanol fermentation (either sugarcane juice or molasses), integrating first and second ethanol generation (E1G2G). Dias et al. (2012) found that an integrated plant represents better environmental profile and economic metrics in comparison with a stand-alone plant. However, at the present process development stage is highly recommended to set up an operational protocol for fermentation using only C6 liquor. This is because the implementation of this configuration is feasible during off-season or for initial implementation of E2G plant. First, the fermentability of C6 liquor was evaluated in batch mode to support the process development of the current mode as Melle-Boinot fermentation.

Please cite this article as: Yamakawa C., Rojas S., Geraldo V., Rivera E., Herrera W., Bonomi A., Rossell C., Maciel Filho R., 2016, Ethanol fermentation of cellulosic hydrolysate from sugarcane bagasse to develop stand-alone second generation ethanol plant, Chemical Engineering Transactions, 50, 163-168 DOI: 10.3303/CET1650028

## 2. Materials and methods

### 2.1 Microorganism and substrate

The microorganism used was *Saccharomyces cerevisiae*, an unclassified strain cultivated in the Development Bioprocess Laboratory at CTBE and obtained from the Faculty of Food Engineering/ State University of Campinas, originally coming from an industrial ethanol distillery. A unique cell propagation was provided to attend the yeast mass required for all ethanol fermentations. The stock culture maintained at -80 °C with 30% (v/v) of glycerol was activated in a liquid medium composed with 10 kg/ $m^3$ of yeast extract, 20 kg/ $m^3$ of peptone and 20 kg/ $m^3$ of glucose, incubated at 33 °C, 250 rpm of agitation during 24 h in the orbital shaker Innova 44 (New Brunswick, USA). Then, an aliquot was transferred to a new medium with 5.0 kg/ $m^3$ of DAP and 80 kg/ $m^3$ of sugars in terms of TSAI (total sugars as inverted that represents sum of sucrose divided per 0.95, glucose and fructose) from sugarcane molasses and juice and it was incubated in the same previous conditions during 12 h. After that, cells were recovered in the high-performance centrifuge Avanti J-26 XP (Beckman Coulter, USA) with rotor JLA-16.250 at 5,509 x g, 10 °C for 15 minutes. The pellet was diluted with sterilized potable water and this suspension was inoculated in the bioreactor Bioflo 115 of 2 L (New Brunswick, USA) with initial cell concentration approximately 1 kg/ $m^3$. The growth phase were in fed-batch, aerated, and limiting carbon feed to less than 18 kg TSAI/ $m^3$.h, to minimize ethanol production through Crabtree effect (Barford & Hall 1979). The agitation and airflow were controlled by oxygen control in cascade mode with dissolved $O_2$ concentration below 60% of saturation. The growth medium contained 180 kg TSAI/ $m^3$ and 4.5 kg/ $m^3$ of DAP. After sugars uptake, the fermented medium was pumped aseptically to flask using peristaltic pump 520U (Watson Marlon, USA). The medium was centrifuged for cells recovery in the same centrifuge used before using rotor JLA-9.100 at 13,261xg, 20 °C for 20 minutes. The cells were re-suspended with sterilized potable water and kept refrigerated at 5 °C until to use for ethanol fermentations.

The sugarcane substrate used for growth medium was formulated with 79% (w/w) of TSAI from sugarcane juice and 21% (w/w) of TSAI from sugarcane molasses. Physicochemical clarifying treatment was applied according to Coopersucar (1987). The same treatment was applied for C6 liquor for ethanol fermentations.

The production of C6 liquor consisted of hydrothermal pretreatment of bagasse performed in a 350 L stirred reactor (Pope Scientific, USA) with 10% (w/v) of solids at 190°C during 10 min. After that, the material was discharged to a Nutshe filter (Pope Scientific, USA) where the liquid phase rich in pentose was separated and discharged. The solid phase, cellulignin, was washed in the basket centrifuge (Ferrum, Switzerland) with water until pH reach 5.0. Then, cellulignin was hydrolyzed with 10 FPU/ mL of commercial enzyme complex in a 20 L horizontal tumbling reactor at 50°C, 20% of solids for 48 hours, pH adjustment at 4.8 with $H_2SO_4$ and NaOH resulting in a slurry that was centrifuged in basket centrifuge. The liquid phase rich in glucose was recovered, clarified and sterilized at 121 °C for 15 min.

### 2.2 Ethanol fermentation

A bioreactor Bioflo 115 of 1.5 L (New Brunswick, USA) at 33 °C and 150 rpm was used for all the experimental runs. The first group (A) of ethanol fermentations carried out sequentially with cell recycle and acid treatment adjusted at pH 2.5 with sulphuric acid during 30 min between each cycle. These fermentations aimed to evaluate fermentability of C6 liquor. This set of fermentations suggested the requirement of nutrient supplementation. Thus, three sources of nitrogen were chosen (urea, molasses and DAP) to evaluate according to a full factorial experimental design, which represents the second group of fermentations (B). This group corresponds to 16 unique fermentations with fresh yeast previously propagated and kept in suspension under refrigeration. Before each fermentation, an acid treatment was conducted adjusting pH at 2.5 with sulphuric acid during 30 min. The limits of the full factorial experimental design were in kg/ $m^3$: (0; 1) for DAP, (0; 0.65) for urea, and (0; 16.65) for sugarcane molasses. The upper limit of DAP was defined based on previous results from successive fermentations (group A), which shows 1 kg/ $m^3$ was an enough concentration. The urea limit was defined at 0.65 kg/ $m^3$ in the case of urea compete with DAP in term of nitrogen. The upper limit of molasses followed the criteria to contribute TSAI increasing in a maximum of 15% (w/w) keeping the fermentation mostly with C6 liquor.

### 2.3 Analytical procedures

The cellular viability and budding were analyzed by staining with methylene blue (Lee et al., 1981), counted using Neubauer chamber in an optical microscopy Eclipse CI-S (Nikon, Japan) for the final sample. The results of high-performance liquid chromatography (HPLC) were used to determine fermentative parameters and to acquire profiles data for mathematical modeling. Agilent Infinity 1260 with IR detector 50 °C, Aminex column HPX-87P 300 mm x 7.8 mm at 60°C and 0. 5 mL/min of ultrapure Milli-Q water as eluent phase determined sucrose, glucose and fructose. Dionex Ultimate 3000 with IR detector Shodex RI-101, Aminex column HPX-87H 300 mm x 7.8 mm at 50°C and 0.5 mL/min of sulphuric acid 5 mM as eluent phase determined ethanol, acetic acid, latic acid and glycerol. Agilent Infinity 1260 with UV detector at 274 nm,

Acclaim 120 - C18 150 x 4.8 mm at 25°C and 0.8 mL/ min of acetonitrile with water (1:8) with 8% of acetic acid as eluent phase determined furfural and hydroxymethylfurfural. The dry cell concentration was determined gravimetrically after centrifuging, washing two times with Milli-Q water and drying at 80 °C until constant weight in the analytical balance.

**2.4 Determination of the fermentation parameters**

The following parameters were determined:

Specific ethanol productivity = $(\Delta E) / (Q_w t)$ [kg/ $m^3$. h] (1)

Gay-Lussac yield ($Y_{P/S}$) = $(\Delta E) / (\Delta S) / 0.511$ (Lavarack, 2003) (2)

By-products yield (Y) = $100/ [0.511 (1+ K_{CO2} + K_X + K_G + K_{AC} + K_{TSAI\ residual})]$ (Finguerut 2006) (3)

Ratio of carbon dioxide per ethanol ($K_{CO2}$) = $(0.9565 + 1.33K_X)/ (\Delta E)$ (4)

Ratio of cell per ethanol ($K_X$) = $(\Delta Y) / (\Delta E)$ (5)

Ratio of glycerol per ethanol ($K_G$) = $(\Delta G) / (\Delta E)$ (6)

Ratio of acid as lactic acid per ethanol ($K_{AC}$) = $(\Delta AC) / (\Delta E)$ (7)

Ratio of TSAI residual per ethanol ($K_{TSAI\ residual}$) = (TSAI final) / $(\Delta E)$ (8)

where $\Delta i$ is the difference between final and initial concentration of compound i; $Q_w$ the final volume of wine with cell; t is the fermentation time.

## 3. Mathematical modeling

The evaluation of inhibitions through mathematical modelling was based on Rivera et al. (2013) using the same kinetic model where the following parameters are fixed: $m_s = 0.2$, $m_p = 0.1$, $K_s = 4.1$ kg/$m^3$, m = 1.0 and n = 1.5 (de Andrade et al. 2013). Differently of the referenced work, the mass balance equations are a batch model described by Eq(9) to Eq(11).

$$r_x = \mu_{max}\left(\frac{S}{K_s+S}\right)\exp(-K_i S)\left(1-\frac{X}{X_{max}}\right)^m\left(1-\frac{P}{P_{max}}\right)^n X \quad (9)$$

$$r_s = \frac{r_x}{Y_x} + m_s X \quad (10)$$

$$r_p = Y_{p/x} r_x + m_p X \quad (11)$$

## 4. Results and discussion

The Table 1 shows compositions of cellulosic hydrolysate used in fermentations (A) and for 16 runs of experimental design (B). The acetic acid, hydroxymethylfurfural (HMF) and furfural concentrations are far to cause inhibition during ethanol fermentation (Taherzadeh 1999; Palmqvist et al. 1999, Delgenes et al.1996, and Rumbold et al. 2009). Besides these compounds, it is noticeable the presence of phenols, which are not measured. However, it is expected a low concentration of phenolic since the hydrothermal pretreatment cause few sugars and lignin degradation, moreover the washing of cellulignin removes soluble fractions from lignin (Santucci et al. 2015). The initial cell concentration was set according to glucose concentration in order to obtain representative profiles curves of glucose or TSAI consumption and ethanol production.

*Table 1: Composition of cellulosic hydrolysate (C6 liquor)*

| C6 liquor | Glucose (kg/ $m^3$) | Xylose (kg/ $m^3$) | Acetic acid (kg/ $m^3$) | HMF (kg/ $m^3$) | Furfural (kg/ $m^3$) |
|---|---|---|---|---|---|
| A | 23.944 | 1.201 | 0.123 | - | 0.001 |
| B | 38.215 | 3.242 | 0.251 | 0.019 | 0.001 |

The Figure 1 shows the specific ethanol productivity, yield, cell viability and budding. The recovery of specific ethanol productivity and yield from fermentation #4 is attributed to nutrient supplementation with DAP and achieved stationary values from fermentation #5, average specific ethanol productivity of 1.341 kg/ $m^3$.h and average yield of 90.86%. The percentage of cell viability and budding remained above 90% and 1%, respectively. Figure 2 presents the pH during the ethanol fermentation. The first fermentation #1 exhibited initial pH of 3.68 and after 4 h was 2.7. It was decided to adjust to 4.5 with addition of KOH solution, since the favourable range is 3.8 to 4.2 (Andrietta et al. 2011). The decrease in the pH can be attributed to low salt composition in the liquor, which promotes buffering characteristics as in the juice and molasses of sugar cane (Souza, 1995). After 12 h of fermentation, the wine was centrifuged to recovery the cells to start new fermentation #2. Firstly, the pH was adjusted at 4.5 with KOH. However, with only 16 hours of fermentation converted half the glucose into ethanol and the pH was 2.87. Probably, the reduction of conversion rate is due to a medium deficiency. The fermentation #3 started with the addition of KOH to adjust pH to 5.5 at the beginning and addition of 1.0 g DAP/ L after 4.5 h to supplement liquor with nitrogen and phosphorus. This results in fermentation progress with recovery of yield and productivity. In subsequent fermentations #4 until

#8 were added 1 kg DAP/ $m^3$ at the beginning leading to in total conversion of glucose into cells, ethanol and carbon dioxide.

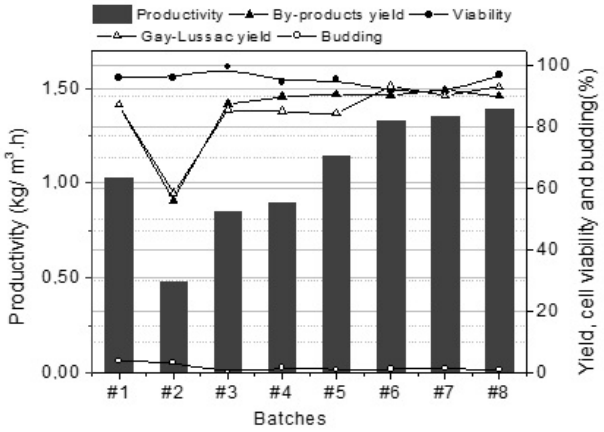


*Figure 1: Ethanol fermentation performance.*

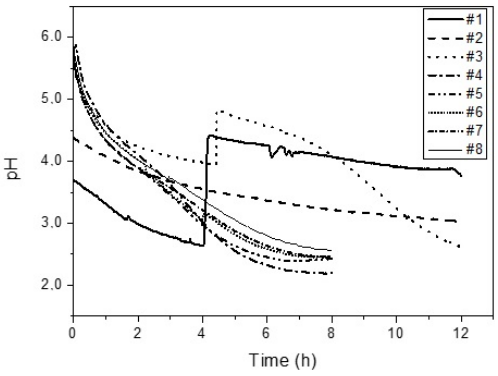


*Figure 2: Profiles of pH during ethanol fermentation.*

The mathematical modelling was evaluated for the last three fermentations, and according to Figure 1 reached stationary fermentation. The results of estimation kinetic parameters presented in Table 2 are compared to Rivera et al. (2013), whose applied the same yeast strain using substrate from sugarcane juice in the fed-batch mode with cell recycle. It was found that the value of $\mu_{max}$ is lower and its likely due to C6 liquor poor characteristics in terms of nitrogen and salts that contribute for yeast maintenance and growth. The initial concentration of substrate and ethanol also influenced in the other parameters, especially $P_{max}$ and $X_{max}$. From Figure 3, it can be seen that the model predictions were particularly good based on Residual Standard Deviation (RSD%) = 1.91 and $R^2$= 0.97 that correspond to the sum of the three experiments.

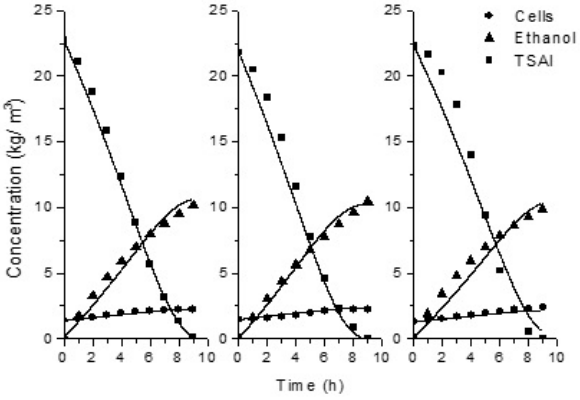


*Figure 3: Experimental measures (solid symbols) and model prediction (solid lines).*

*Table 2: Estimated parameters values*

| $\mu_{max}$ ($h^{-1}$) | $P_{max}$ (kg/ $m^3$) | $X_{max}$ (kg/ $m^3$) | $Y_{p/x}$ (kg/ kg) | $Y_x$ (kg/ kg) | $K_i$ ($m^3$ /kg) |
|---|---|---|---|---|---|
| 0.11 | 125.5 | 79.3 | 9.69 | 0.0048 | 0.00999867 |

Based on promising results obtained with DAP supplementation, it has been proposed the assessment of other competitive nutritional sources such as urea and molasses. The DAP is a fertilizer used in agriculture, but is less economically competitive than urea. According to Villalba et al. (2014), nitrogen fertilizers more consumed in Brazil are: urea (45% N), monoammonium phosphate (9% N), ammonium sulphate (20% N), ammonium nitrate (32% N), DAP (16% N), and other complex as nitro-calcium aqua ammonia. The molasses contains fermentable sugars, potassium, calcium, magnesium, trace minerals, biotin, pantothenic acid, thiamine, and low amount of nitrogen (Zabriskie 1980). The addition of these components serves as a buffer avoiding sudden drop in pH. The price accessed in August/ 2015 of DAP and urea (Index Mundi) are USD 464/t and USD 273/t, respectively. The molasses price is USD 336/t (Shapouri & Salassi 2006). The use of DAP, urea and molasses could increase the ethanol cost between 0.021 and 0.295 USD/L. Thus, an assessment of how to minimize the incorporation of these components is an important study to be performed.

The results of experimental design (B) are presented in Figure 4, the pH profiles during fermentation exhibit low values when compared to the current fermentation of sugarcane juice/ molasses, which the common value of final wine is 3.5. However as presented at Figure 5, a pH decreasing not influenced yeast viability (above

90%) and budding. Besides, the yields calculated from Eq(2) and Eq(3) are similar in average of 87%, and they are not influenced by sources of variables. In ethanol fermentation is desirable pH around 3.0 and 3.5 to preserve cellular membrane and to avoid bacterial contamination; this policy corresponds to molasses and DAP use.

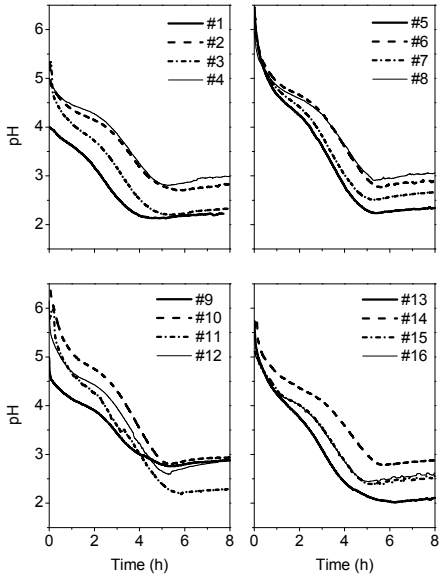


*Figure 4: Profiles of pH during fermentation.*

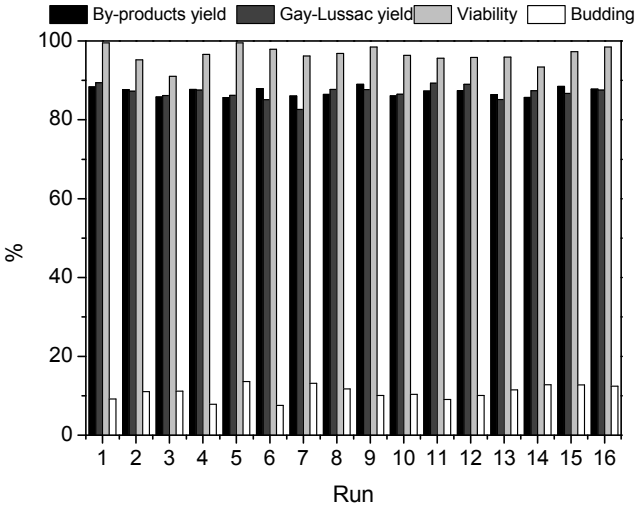


*Figure 5: Performance of ethanol fermentation.*

Table 3 shows the results of ANOVA analysis considering polynomial model. The F-test of $F_{9,6}$ is greater than listed value; therefore the model is significant. The second F-test of $F_{5,1}$ is less than listed value indicating that –the model is well adjusted. The statistical analysis of the data obtained in the design was carried out using the software Statistica (version 12, Statsoft Inc., Tulsa, OK). According to Figure 6, only molasses is significant at 95% confident level.

*Table 3: Results of ANOVA analysis*

| Source of variation | Sum of squares | Degrees of freedom | Average squares |
|---|---|---|---|
| Regression | 0.4858 | 9 | 0.053973 |
| Residual | 0.0697 | 6 | 0.011612 |
| Lack of fitting | 0.0697 | 5 | 0.013935 |
| Pure error | 0.0012 | 1 | 0.001160 |
| Total | 0.5554 | 15 | |
| R-square | 87.46 | | |
| F-test $F_{9,6}$ | 4.6 | $F_{9,6}$ listed (95%) | 4.1 |
| F-test $F_{5,1}$ | 12.0 | $F_{5,1}$ listed (95%) | 234 |

The molasses requirement of 8.32 kg/m$^3$ corresponds to 0.147 USD/L. With this, the feasibility of the E2G integrated to E1G technology is reinforced. However, the operation as stand-alone E1G can be performed given a competitive price of molasses. The next step is to evaluate the concentration of C6 liquor during fermentation with cell recycle to obtain fermented mash with usual ethanol content of 70 g/ L.

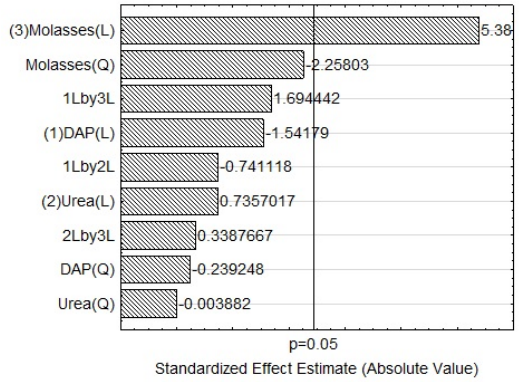


*Figure 6: Pareto chart of standardized effects on productivity.*

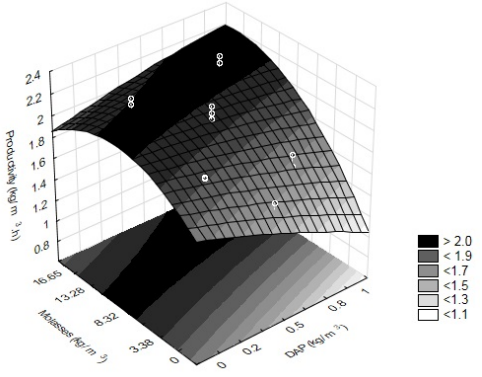


*Figure 7: Response surface for productivity as a function of molasses and DAP.*

## 5. Conclusions

A nutrient supplementation is required for fermentation of just C6 liquor in the stand-alone E2G plant. The sugarcane molasses is promising source due to buffering capacity and nutritional aspect. Only DAP and urea are insufficient to supply the demand of macro and microelements.

**Acknowledgments**

This work was supported by FAPESP (Process number 11/51902-9). We are grateful to CNPEM and CTBE for helpful assistance on facilities and knowledge.

**Reference**

Andrietta, M., Andrietta SR & Stupiello, E., 2011. Bioethanol what has Brazil learned about yeasts inhabiting the ethanol production processes from sugar cane?, 67–84, DOI: 10.5772/16572.

Barford, J.P. & Hall, R.J., 1979. An Examination of the Crabtree Effect in Saccharomyces cerevisiae: the Role of Respiratory Adaptation. *Journal of General Microbiology*, 114(2), 267–275, DOI: 10.1099/00221287-114-2-267.

Canilha, L. et al., 2012. Bioconversion of Sugarcane Biomass into Ethanol: An Overview about Composition, Pretreatment Methods, Detoxification of Hydrolysates, Enzymatic Saccharification, and Ethanol Fermentation. *Journal of Biomedicine and Biotechnology*, 1–15, DOI: 10.1155/2012/989572.

Coopersucar, Cooperativa de produtores de cana, açúcar e álcool do estado de São Paulo, 1987. Fermentação. São Paulo, 71-74 (in Portuguese).

De Andrade, R.R. et al., 2013. Kinetics of ethanol production from sugarcane bagasse enzymatic hydrolysate concentrated with molasses under cell recycle. *Bioresource Technology*, 130, 351–359, DOI: 10.1016/j.biortech.2012.12.045.

Delgenes, J.P., Moletta, R. & Navarro, J.M., 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae. *Enzyme and Microbial Technology*, 19(3), 220–225, DOI: 10.1016/0141-0229(95)00237-5.

Dias, M.O.S. et al., 2012. Integrated versus stand-alone second generation ethanol production from sugarcane bagasse and trash. *Bioresource Technology*, 103(1), 152–161, DOI: 10.1016/j.biortech.2011.09.120.

Finguerut, J. I., 2006. I Workshop tecnológico sobre obtenção de etanol. Projeto PPPP Etanol – Universidade de São Paulo – Escola de Engenharia de Lorena, Lorena, SP, Brasil.

IndexMundi, 2015. Commodity Prices <http://www.indexmundi.com/> accessed 26.11.2015

Lavarack B. P., 2003. Estimates of ethanol production from sugar cane feedstocks*. Proc. Aust. Soc. Sugar Cane Technol.,* 25.

Lee S. S., Robinson F. M., Wang H. Y., 1981. Rapid determination of yeast viability. Biotechnology and Bioengineering Symposium, 11,641-649.

Palmqvist, E., Almeida, J.S. & Hahn-Hägerdal, B., 1999. Influence of furfural on anaerobic glycolytic kinetics of saccharomyces cerevisiae in batch culture. *Biotechnology and Bioengineering*, 62(4), 447–454, DOI: 10.1002/(SICI)1097-0290(19990220)62:4<447::AID-BIT7>3.0.CO;2-0.

Rivera, E.C. et al., 2013. A Procedure for Estimation of Fermentation Kinetic Parameters in Fed-Batch Bioethanol Production Process with Cell Recycle. *Chemical Engineering Transactions*, 32, 1369–1374, DOI: 10.3303/CET1332229.

Rumbold, K. et al., 2009. Microbial production host selection for converting second-generation feedstocks into bioproducts. *Microbial cell factories*, 8, 64, DOI: 10.1186/1475-2859-8-64.

Santucci, B.S. et al., 2015. Autohydrolysis of Hemicelluloses from Sugarcane Bagasse During Hydrothermal Pretreatment: a Kinetic Assessment. *BioEnergy Research*., DOI: 10.1007/s12155-015-9632-z.

Shapouri, H. & Salassi, M., 2006. The economic feasibility of ethanol production from sugar in the United States. USDA report, (July), 69.

Souza, J. S. I.; Peixoto, A. M. & De Toledo, F. F., 1995. Enciclopédia Agrícola Brasileira, São Paulo, EdUSP, vol. 4 I-M, 468, ISBN 853140719-2.

Taherzadeh, M.J., Niklasson, C. & Lidén, G., 1999. Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation. *Bioresource Technology*, 69(1), 59–66, DOI: 10.1016/S0960-8524(98)00169-2.

Villalba, G. H. A. et al., 2014. Fertilizantes nitrogenados: Novas tecnologias. IPNI, Boletim Informações Agronômicas, 148.

Zabriskie, D. W. 1980. *Traders' guide to fermentation media formulation*. Fort Worth, Tex, Traders Protein Division of Traders Oil Mill.