CHEMICAL ENGINEERING TRANSACTIONS
VOL. 38, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Taj Keshavarz

ISBN 978-88-95608-29-7; **ISSN** 2283-9216
DOI: 10.3303/CET1438082

Second Generation Ethanol Production Using Crude Enzyme Complex Produced by Fungi Collected in Brazilian Cerrado (Brazilian Savanna)

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Bioconversion of agro-industrial residues has gained much focus worldwide as effective strategy to reduce environmental pollution and improve ethanol production. Therefore, the Brazil as one of the world's biggest agro-industrial residues producer can use residues such as rice straw, sugarcane bagasse and others to produce cellulolytic enzymes for enzymatic hydrolysis for second-generation bioethanol production.
The objective of this work was to produce cellulolytic enzymes and use these enzymes to produce second generation ethanol from sugarcane bagasse. Twelve cellulolytic enzymes micro-organism from Brazilian Cerrado (Savanna) were tested to produce crude enzyme extract by solid state fermentation and the enzyme complexes were used to produce ethanol from exploded sugarcane bagasse using *Saccharomyces cerevisiae* Y904.
All crude enzymatic complex used were able to produce ethanol from sugarcane bagasse, thus suggesting that the Brazilian Cerrado appears to be a promising source of cellulolytic enzymes. The best results were found for 48h of fermentation using crude enzymatic extract produced by *Aspergillus niger* (11.5 g/L of ethanol) and *Mucor racemosus Fresenius* (7.2 g/L of ethanol).

1. Introduction

The depletion of fossil fuels has driven the world to utilize renewable-energy sources such as ethanol based on raw materials rich in complex carbohydrates including starch, oligosaccharides and cellulosic biomass. Generally the ethanol produced from complex carbohydrates follow four process steps: feedstock pretreatment, enzymatic hydrolysis, sugars fermentation, and ethanol recovery (Gómez et al., 2010; Binod et al., 2012; Zanette et al., 2008; Tyea et al., 2012; Silva et al., 2012).
The biomass used for fuel generation include forest by-products, sugarcane bagasse, wood residues, switchgrass, sweet sorghum, waste paper and other common agricultural wastes. Agricultural waste such as sugarcane bagasse is abundant in Brazil and has being widely investigated for its potential for producing of ethanol. Brazilian sugarcane harvest in 2011/12 was 560 million tons and for each ton of sugarcane processed, approximately 250 to 280 kg of bagasse is produced (MAPA, 2012; CTC, 2012).
Acknowledging the increasing demand for second generation ethanol the use of crude enzyme complex rich in cellulase enzymes looked at as a viable alternative capable of reducing the cost of generation ethanol and the SSF emerges as an important way to enzyme production in situ (Thomas et al., 2013; Rocha et al., 2013; Aswathy et al., 2010).
In order to improve heat and mass transfer, the SSF process has been performed using various bioreactors types (packed-beds, rotating drums, gas-solid fluidized beds and various stirred bioreactors) and crude enzyme complex is obtained at the end of SSF by washing the solid fermented medium (Mitchell et al., 2000; Mitchell et al., 2003; Lever et al., 2010).
Cellulolytic enzymes of fungi have been researched in various environments including Brazilian savannas (Cerrado) ecosystems that occupy 22% of the Brazilian territory and have approximately 2 million square kilometers. It is one of the largest biomes in the world with great potential for commercial enzymes which

Please cite this article as: Fischer J., Lopes V., Santos E.F.Q., Coutinho Filho U., Cardoso V., 2014, Second generation ethanol production using crude enzyme complex produced by fungi collected in brazilian cerrado (brazilian savanna), Chemical Engineering Transactions, 38, 487-492 DOI: 10.3303/CET1438082

have received less attention as compared to other biomes (Ratter et al., 1997; Marchant, 2010; Fischer et al., 2013).

In this context, *Aspergillus, Penicillium, Trichoderma, Chaetomium*, *Mucor* and other fungi have been reported by several researchers as producers of cellulolytic enzymes by Solid state fermentation (SSF) and the most promising source to broaden the range of enzymes essential to advances in enzyme technology including ethanol production (Buzzini et al., 2002; Thomas et al., 2013; Fischer et al., 2013; El-Said e Saleem, 2008).

Thus, the present work fungi collected in the tropical savanna of Brazil (Cerrado) was evaluated in terms of quality of crude enzyme complex produced via solid state fermentation. Fungal species were identified and crude enzyme complex produced were used to produce ethanol from exploded sugarcane bagasse and *Saccharomyces cerevisiae*.

2. Experimental

2.1 **Materials**

Twelve strains, described here as C7, E3, E6 ,E8, F2 ,I1 ,I2 ,I3 ,I4, I5, I6 and I7, obtained from secondary screening 66 evaluated in previous work (Fischer et al., 2013), were kept on slants of Czapek agar stored at 5°C and routinely subcultured onto same medium with the following composition (g/L): $NaNO_3$ 2.0; K_2HPO_4 1.0; $MgSO_4$ 0.5; KCl 0.5 ;$FeSO_4$ 0.01; glucose 20.0; agar 20.0.

All ethanol fermentations were performed using *Saccharomyces cerevisiae* Y904 obtained as lyophilized ethanol yeast from Mauri Brasil SA (Brazil) (Rocha et al., 2013). The yeast was stored in lyophilized form at 5 ± 1 °C for the entire study period.

The solid biomass used in the fermentations was rice byproduct milled and sieved through 1.8 mm mesh, and exploded sugarcane bagasse in low severity condition (Jollez et. al., 1993) obtained in a batch system at temperature 191°C (8 min), from Sugarcane Research Center-CTC (São Paulo, Brazil). All biomass used was stored at 5 ± 1 °C for the entire study period.

2.2 **Fermentations**

SSF to produce the enzyme complex were performed for 72 h at 30 ± 1 °C in a 250 mL Erlenmeyer flask containing 10^7 to 10^8 cells/g of fungi, 40 g of solid medium with 24 g of rice bran and 16 g of exploded sugarcane bagasse. Crude enzyme complex-extraction medium as Tween 80 (1%) and distilled water (Rocha et al., 2013).

The ethanol fermentations (35 °C, 48 h) were performed in 250 mL Erlenmeyer with 100 mL of crude enzyme complex resulting from the extraction of enzymes from SSF, 20 g of exploded sugarcane bagasse, 3 g of *Saccharomyces cerevisiae* Y904. This study was conducted in three phases. In the first ethanol production from crude enzyme complex provided by all strains were evaluated. In the second phase ethanol production from association between crude enzymes complexes belonging to two different strains (*Mucor racemosus Fresenius and Aspergillus niger*) were evaluated (Table 1). In the third a fed-batch ethanol production were evaluated: after 48h of fermentation the undegraded solid material was removed by filtration through glass fibre filter, the supernatant was returned to the Erlenmeyer along with an additional 20 g substrate (of exploded sugarcane bagasse).

Table 1: Crude enzyme complex from Mucor racemosus Fresenius and Aspergillus niger

	(%) Enzyme complex						
Experiment	A	B	C	D	E	F	G
Enzyme complex from M. racemosus Fresenius	0	25	35	50	65	80	100
Enzyme complex from complex A. niger	100	75	65	50	35	20	0

2.3 **Analytical methodology and strain identification**

All fermentations were analyzed for ethanol production using high performance liquid chromatography (Shimadzu model LC-20A Prominence, Ca Supelcogel column) where the substances were detected by refractive index and identification of strains at species level based on morphological and biochemical tests (Alves-Prado et al., 2010; Barnett and Hunter, 1999).

3. Results and discussions

Figure 1 presents the ethanol concentration at 35 °C after 48 h of fermentation with media composed of 200 g/L of steam-exploded sugarcane bagasse obtained from crude enzyme complex provided by twelve

strains and the results of identification of strains at species level based on morphological and biochemical tests. According to this results, all crude enzymatic complex used were able to produce ethanol from sugarcane bagasse and the most promising species with regards to the production of an enzymatic complex in order to produce ethanol were *A. niger* (11.5 g/L of ethanol) and *M. racemosus Fresenius* (7.2 g/L of ethanol).

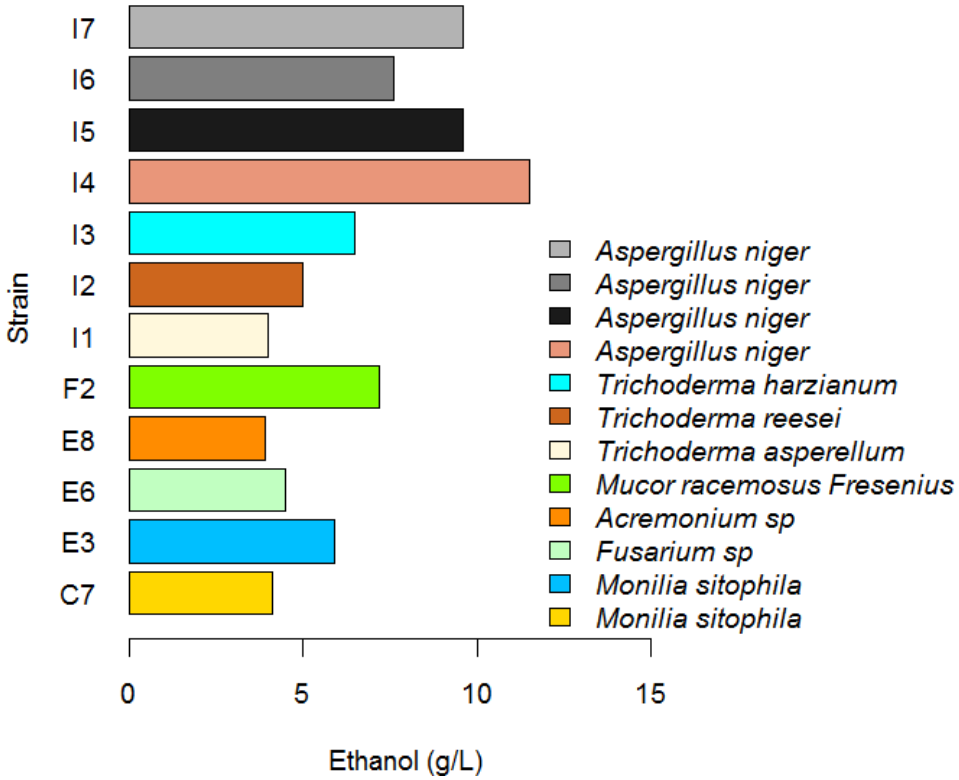

Figure 1: Ethanol concentration for the twelve strains after 48 h of fermentation and the results of identification of strains

Based on results found in Figure 1, the species *M. racemosus Fresenius* and *A. niger* were selected for further experiments. Ethanol concentrations for association between enzymes complexes produced by these species were evaluated for the formulations A-G containing crude enzyme extract from *M. racemosus Fresenius* and *A. niger* (Figure 2). A comparison between A-G shows a significant increase in the ethanol concentration when the crude enzyme extract of *A. niger* is increased from 0 to 100%.

The ethanol conversion (g ethanol/ g substrate) found for *M. racemosus Fresenius* (0.34 g/g) and *A. niger* (0.54 g/g) are in agreement with results published by literature (Cardone et al., 2014; Gupta et al., 2009; Ofori-Boateng et al., 2014) suggesting the strains used appears to be a promising source of cellulolytic enzymes.

The ethanol concentrations produced with feed-batch exploded sugarcane bagasse are shown in Figure 3. After 48 h of fermentation bagasse was added and the ethanol concentration increased from 5.5 to 15.6 g/L (*M. racemosus Fresenius*) and 10.1 to 16.4 g/L (*A. niger*), revealing that it is possible to achieve cellulosic ethanol concentrations by sequential batch addition of substrate. The maximum concentration achieved in fed-batch (1.6 % w/v) was not optimal, ethanol concentrations above 4 % (w/v) are needed to reduce distillation costs (Zacchi and Axelsson, 1989), but the SSF and ethanol fermentations conditions must further optimized for ethanol production.

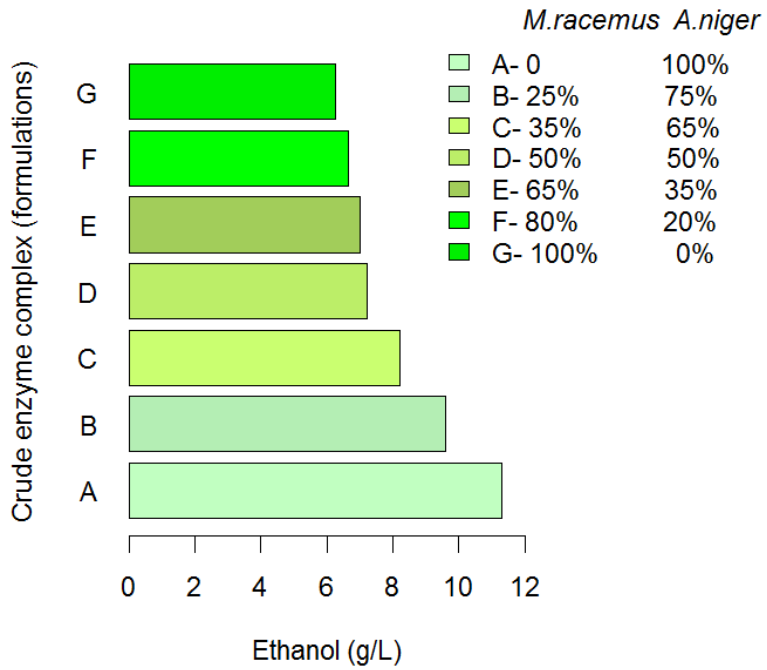

Figure 2: Ethanol concentration for association between crude enzymes complexes belonging to Mucor racemosus Fresenius and Aspergillus niger

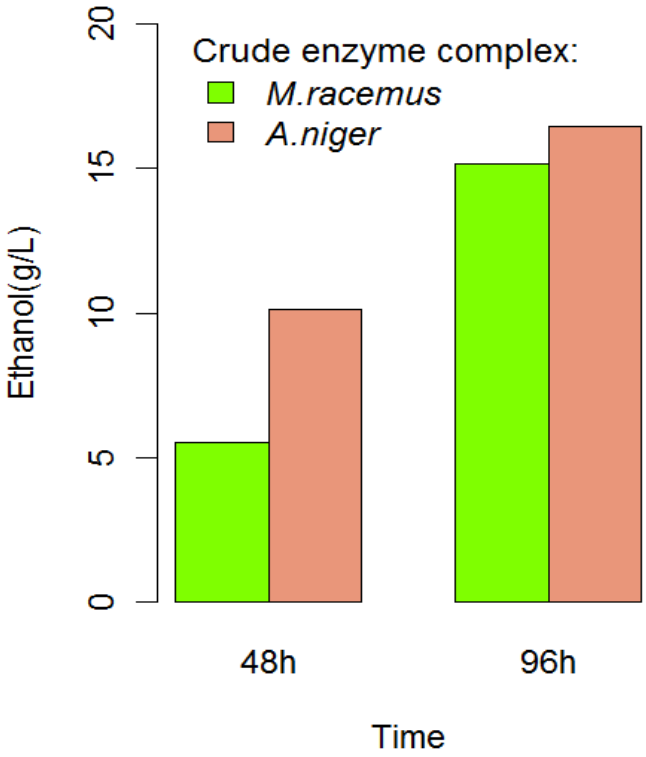

Figure 3: Ethanol concentration for association between crude enzymes complexes belonging to Mucor racemosus Fresenius and Aspergillus niger

4. Conclusions

From the experiments performed in this work, the following can be concluded:
a) All strains tested were found to be a very promising micro-organism with regards to the production of an enzymatic complex in order to degrade cellulose and produce ethanol.
b) Under the conditions in this study, the use of additional exploded sugarcane bagasse loadings improved the yield of ethanol compared to the yield obtained in a single use of exploded sugarcane bagasse.
c) Further improvement in the ethanol fermentation may be obtained by employing suitable nutrient and enzyme complex feeding strategies to increase ethanol concentration above 4 % (w/v).

Acknowledgements

The authors wish to thank the CNPq (Edict 046/2009, Project 550940/2010-3), CAPES and FAPEMIG - Brazil – for the financial support and the CTC by the partnership in joint studies related to ethanol production.

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