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Xylanase Production by *Trichoderma reesei* Rut C-30 on Rice Straw

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Abstract

Xylanase production of *Trichoderma reesei* Rut C-30 was examined at different initial pH values (4.8, 5.9, and 7.0) on rice straw in shake flasks, and in a fermentor, for the best pH condition. Enzyme performance was tested on ammonia-treated dwarf elephant grass. The maximum xylanase activities, 92 and 122 IU/mL, were obtained at pH 4.8 in the shake flasks and fermentor, respectively, in which good growth of the fungus was observed during the first 24 h and consumption of proteins dissolved from the rice straw caused the pH to rise later to values between 6.4 and 6.7 (optimal for xylanase production). The xylanases from *T. reesei* were as effective as Multifect XL, a commercial enzyme preparation, in hydrolyzing ammonia-treated elephant grass.

Index Entries: Xylanase; *Trichoderma reesei*; rice straw.

Introduction

Potential uses of lignocellulosics are increasing. The development of green technologies to use them usually includes several steps such as enzyme production, substrate treatment, enzymatic hydrolysis of the substrate, fermentation of hydrolysis products, and recovery of fermentation products *(1)*. Availability of cellulases and xylanases plays a very important role in achieving this goal.

The capability of *Trichoderma reesei* strains to produce active and relatively stable cellulases and xylanases is well known *(2,3)*, but it is still under active research because many factors affecting production and enzyme activ-

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ity are not completely understood *(4,5)*. It has been reported that xylanase production by *T. reesei* Rut C-30 is favored at pH values near neutrality *(6,7)*, whereas cellulase production has a broader range *(5,6)*. However, the performance and characteristics of the system are greatly influenced by the selection of the substrate. In the present work, rice straw was chosen for enzyme production because it is the third most abundant residue in Venezuela *(8)*. The objectives of this work were to evaluate the effect of initial pH on xylanase production by *T. reesei* Rut C-30 on rice straw as well as its hydrolytic performance on ammonia-treated dwarf elephant grass, in comparison with the performance of an available commercial xylanase.

The results of this research will be used by the Technological Park of the University of Zulia (Venezuela) in the development of an ammonia treatment technology.

Materials and Methods

Fermentation Substrate

Rice straw from medium grain grown in Zulia State (Venezuela) was used as the substrate in all of the fermentations. A mixture of 72.4% amorphous cellulose (Solka Floc 40 FCC; International Fiber Corp., North Tonawanda, NY) and 27.6% oat spelt xylan (Sigma, St. Louis, MO), which has amounts equivalent to those of the cellulose and hemicellulose present in rice straw, was used as a control substrate.

Microorganism and Culture Conditions

T. reesei Rut C-30 ATCC 56765 was used. Dilution flasks containing potato dextrose agar (1% dextrose) were used for maintenance and sporulation (5–7 d). Spore suspensions were prepared by adding 10–20 mL of Mandels and Weber's *(9)* mineral medium to the flasks containing spores and stored at 4 ± 1°C until use.

Fermentations

Shake-flask experiments were carried out in 250-mL Erlenmeyer flasks with 1% (w/v) rice straw in 60 mL of Mandels and Weber's *(9)* mineral medium at three initial pH values: 4.8, 5.9, and 7.0 adjusted with 0.1 *N* HCl and 0.1 *N* NaOH. The flasks were kept at 29°C for 120 h at 200 rpm *(7)* in an Innova 4300 orbital air incubator (New Brunswick Scientific, Edison, NJ). The mineral medium and the solids concentration for the control experiment were the same as for the rice straw fermentations; initial pH was adjusted to 4.8. Spore inoculum was adjusted to 1×10^6 spores/g of substrate. All fermentations were carried out in duplicate.

A fermentor experiment was set at the best initial pH condition obtained from the shake-flask experiments. Duplicate fermentations were carried out batchwise in a 4-L Bioflo III model (New Brunswick) with 4 *L* of mineral solution *(9)* at 29°C for 120 h at 150 rpm and aeration of 0.5 vvm.

Dissolved oxygen was controlled at 20% saturation. Antifoaming A agent (Sigma) was used.

Shake-flask sampling was carried out by taking the content of two random flasks every 24 h. For the fermentor study, a 120 to 130-mL aliquot was taken every 24 h. Samples were centrifuged at 12,000*g* for 30 min at 4°C. Enzymatic activity, soluble protein, and pH were determined in the supernatant, and Kjeldahl nitrogen was determined in the solids, which was expressed as milligrams of crude protein/milliliter of culture broth.

Chemical and Enzymatic Analyses

Cellulase and cellobiase activities were determined according to Ghose *(10)*. Xylanase activity was assayed according to Bailey et al. *(11)* but using birchwood xylan (Sigma) instead of birch glucuroxylan. Lowry's method was used to determine soluble protein *(12)*. Kjeldahl nitrogen was determined and used, previous nitrogen material balances, as an estimate of microbial growth at some fermentation periods. The fungus has about 37% crude protein *(13)*. Fiber content of rice straw and dwarf elephant grass was determined by standard fractionation fiber analysis *(14)*.

Treatment of Hydrolysis Substrate

Untreated and ammonia-treated dwarf elephant grass according to Ferrer et al. *(15)*—air-dried, hammermilled to 1 mm, and stored at 4 ± 1°C for 2 yr—was used to test the efficiency on enzymatic hydrolysis of the xylanases produced by *T. reesei* Rut C-30.

Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out at a solids loading of 5% (w/v) in 250-mL Erlenmeyer flasks containing 50 mL of 0.05 *M* citrate buffer (pH 4.8) placed at 50°C at 100 rpm for 24 h in an incubator shaker (Innova 4300; New Brunswick Scientific). Sodium azide was added for preservation (0.15%). Samples (10 mL) were taken at 0, 6, 12, and 24 h and subjected to reducing sugar analysis with the dinitrosalicylic acid method *(16)*. Hydrolysis was performed with the xylanases produced by *T. reesei* Rut C-30 on rice straw and with xylanases produced by *Trichoderma longibrachiatum* (Multifect XL; Genencor, Helsinki, Finland) at 1 IU/g of dry substrate loading. Both preparations were supplemented with 1 IU/g of dry substrate of cellulase (Spezyme CP; Genencor, Rochester, NY) and 5.56 cellobiase units/g dry substrate of cellobiase (Novozym 188, Novo Nordisk, Franklinton, NC).

Results and Discussion

The pH increased in the rice straw fermentations (Fig. 1) and particularly for an initial pH of 4.8 during the first 24 h. The fungus grew heavily during this period, as seen under the microscope, and the growth was better at lower initial pH (4.8 and 5.9), as the crude protein content in the

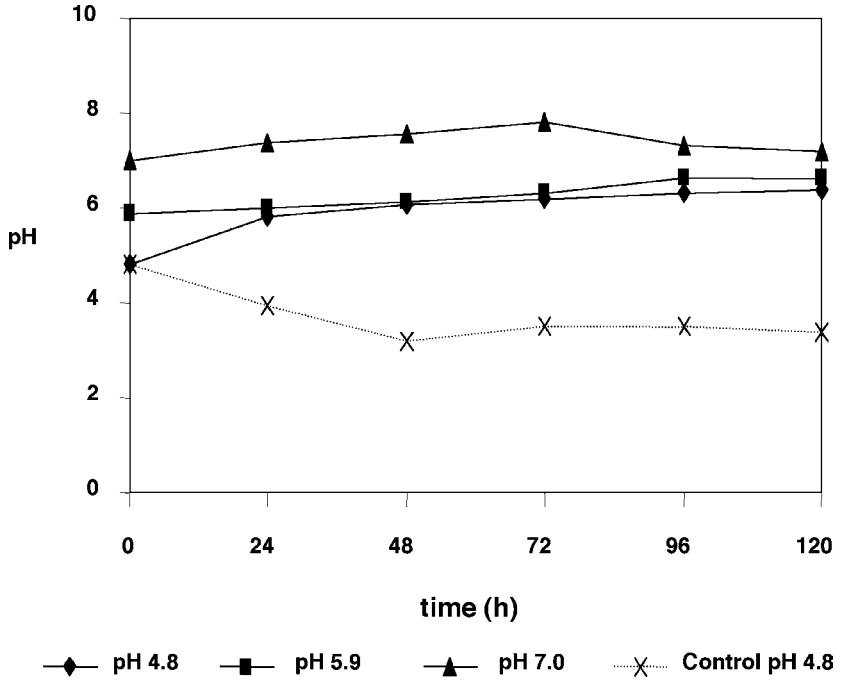

Fig. 1. Variations in pH in shake-flask fermentations of *T. reesei* Rut C-30 on rice straw at different initial pHs and on control substrate.

solid fraction indicates (Fig. 2). Although Fig. 2 shows similar slopes for initial pHs of 4.8 and 5.9, that does not mean that fungus growth was the same since rice straw proteins were being solubilized in the fermentation with the initial pH of 4.8 as pH increased to 5.81 (24 h), which makes about 26% of the protein in the solids to be solubilized, meaning that less protein remains in the solids. In other words, more fungus biomass must be in the solids for the initial pH of 4.8 compared to fermentation with the initial pH of 5.9, in which no more protein is solubilized since pH did not increase much (6.02) by 24 h. The growth of the fungus for the initial pH of 4.8 was as dense as for the control fermentation, in which the pH variation was about 0.88 pH units (from 4.8 to 3.92), indicating high metabolic activity as well, and twice as dense as the fermentation with the initial pH of 5.9. Growth for an initial pH of 7.0 was poor. Crude protein of rice straw was much higher at pH 4.8 (0.6 mg/mL) than at pH 5.9 and 7.0. This is owing to protein solubilization from the straw at pH near neutrality, as has been reported for grasses; the higher the pH, the greater the protein solubilization *(17)*. Since crude protein content of rice straw was 6.75% and crude protein in the solids of a 120-mL sample at the initial pH of 4.8 was 0.6 mg/mL, only 74% of the initial protein was present in the solids; in other words, 26% of the crude protein was solubilized. On the other hand, initial crude protein in solids for the pH 5.9 and 7.0 fermentations was 0.38 mg/mL, which represents a 50% protein solubilization.

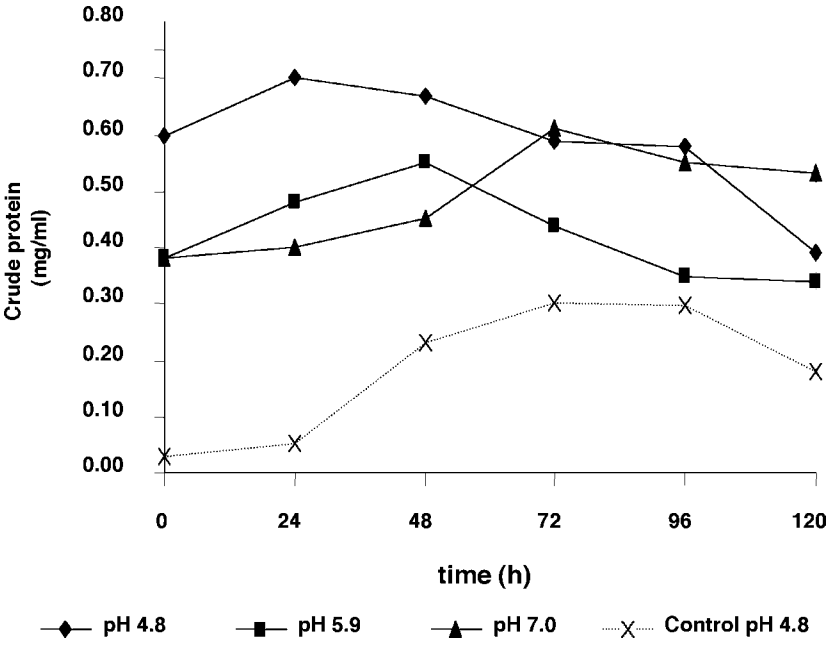

Fig. 2. Crude protein in solids from shake-flask fermentations of *T. reesei* Rut C-30 on rice straw at different initial pHs and on control substrate.

Figure 3, which shows the true protein (rice straw protein and/or extracellular enzymes) released to the medium, confirms that soluble protein was about 26% (estimated by Lowry's method) at the beginning of that fermentation. Soluble protein decreased between 0 and 24 h in both the 4.8 and 5.9 initial pH rice straw fermentations, indicating protein consumption. However, for pH 4.8 the decrease was about 25% (from 0.16 to 0.12 mg/mL), and for pH 5.9 was just 7.1% (from 0.28 to 0.26 mg/mL), representing 100% more consumption of protein (0.04 vs 0.02 mg/mL). It is known that the pH value in *T. reesei* fermentations decreases when the fungus consumes cellulose and does not have either peptone or protein *(18)*. As pH decreased for the control substrate (no protein), one can infer that solubilization of proteins from rice straw (6.75% protein) and subsequent consumption by the fungus were responsible for increasing the pH, likely caused by protonation of amino groups released to the medium. Therefore, since protein consumption is responsible for the increase in pH in the broth (Fig. 1), this explains the greater metabolic activity at the initial pH of 4.8, and thus greater fungus growth. Moreover, the difference observed in protein consumption could be even higher since protein is being solubilized at a greater extent in fermentation with the initial pH of 4.8, hence increasing the soluble protein in the broth. Later, protein in the liquid phase started to increase, mainly owing to enzyme production. After 24 h, pH increased to 5.81, and more rice straw protein was released. This

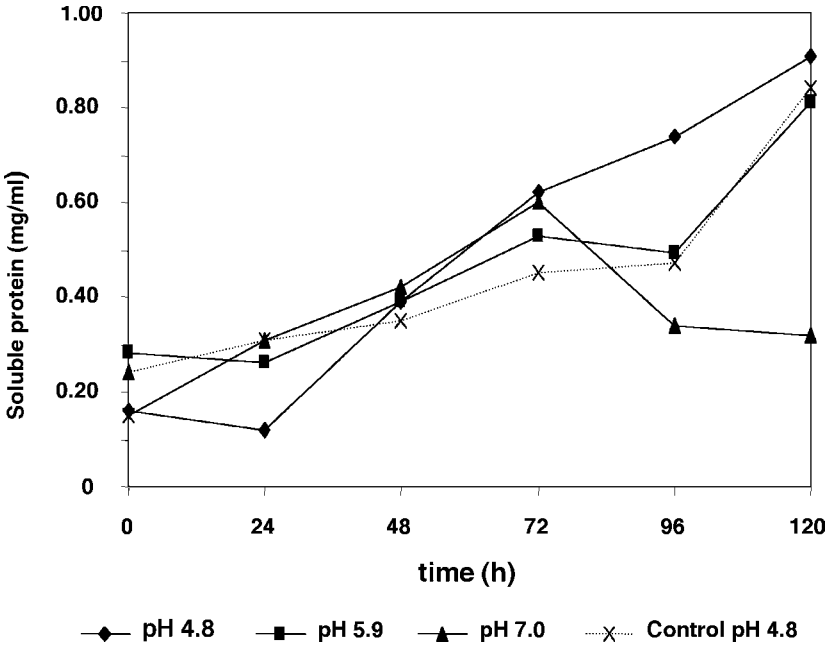

Fig. 3. Soluble protein in shake-flask fermentations of *T. reesei* Rut C-30 on rice straw at different initial pHs and on control substrate.

was greater than the protein provided by fungus growth, and, as a consequence, crude protein decreased.

The highest fungus growth was achieved in the control with a 0.3 mg/mL crude protein content in the solids by 72 h, which represents true growth since there was no protein in the control substrate.

The results presented in Fig. 4 indicate that rice straw (initial pH of 4.8 and 5.9) is better as a substrate for xylanase production than commercial pure cellulose and hemicellulose (control substrate) at the experimental conditions used. Certainly, substrate selection plays a major role in enzyme production. The results in Fig. 4 suggest that the pH developed in the rice straw fermentations was more suitable for xylanase production since it reached values between 6.0 and 7.0, and it has been reported that optimal pH values for xylanase production are between 6.0 and 6.5 *(7)*. On the other hand, the low xylanase production on the control substrate can be explained by pH values far from the optimal at the first stage of the fermentation.

The production of xylanases was higher at lower initial pH, with values of 92, 46, and 8 IU/mL for initial pH values of 4.8, 5.9, and 7.0, respectively (Fig. 4). Since the pH values reached in the fermentations with initial pH values of 4.8 and 5.9 were similar by 24 h, the difference in xylanase production between both fermentations may be assigned to the greater fungus growth in the fermentation starting at pH 4.8 (accelerated pH rise in the first 24 h, intense metabolic activity) since this is considered the optimal pH for

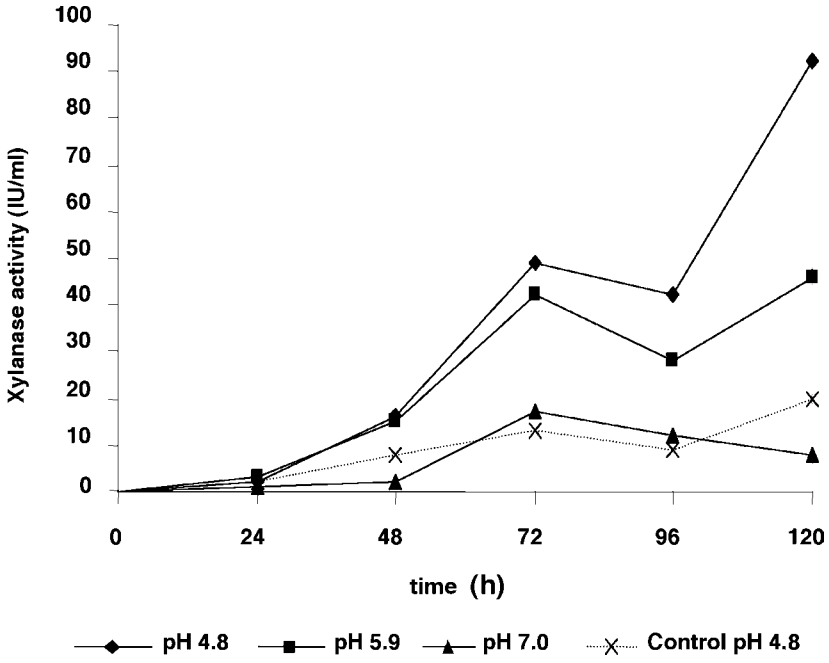

Fig. 4. Xylanase production in shake-flask fermentations of *T. reesei* Rut C-30 on rice straw at different initial pHs and on control substrate.

growth. On the other hand, xylanase production at the initial pH of 7.0 was very low, likely owing to poor growth during the fermentation. By 96 h, xylanase production decreased and later increased again. This behavior is associated with oxygen deprivation in the shake flasks.

Xylanase activity was higher than that reported by Dekker *(19)* for *T. reesei* QM 9414 cultured on sugarcane bagasse (6.4 IU/mL), by Bailey and Poutanen *(20)* for *Aspergillus oryzae* (90 IU/mL) and *A. niger* (49 IU/mL) on wheat bran, and by Gomes et al. *(21)* for *Trichoderma viride* on rice straw (72.4 IU/mL) and newspaper (92.4 UI/mL), although lower than that reported by Gomes et al. *(21)* for *T. viride* (190 IU/mL) on sulfited pulp and by Bailey et al. *(7)* for *T. reesei* Rut C-30 on wheat bran (138 IU/mL).

Cellulase activities measured at 120 h showed a different trend compared to xylanases.

Cellulase activity decreased as the initial pH decreased, giving values of 0.51 IU/mL, 0.29 IU/mL, undetected, and 0.62 IU/mL, for initial pHs of 4.8, 5.7, 7.0, and the control, respectively. When *T. reesei* Rut C-30 was cultured on rice straw and on the control substrate, production of cellulase was lower than that found in other studies with the same microorganism *(22,23)*. Since the cellulase activities of the rice straw fermentation with initial of pH 4.8 and the control fermentation (the same initial pH) were similar, it appears that cellulase activity was not affected by the pH developed during the fermentation, contrary to what was found in xylanase production.

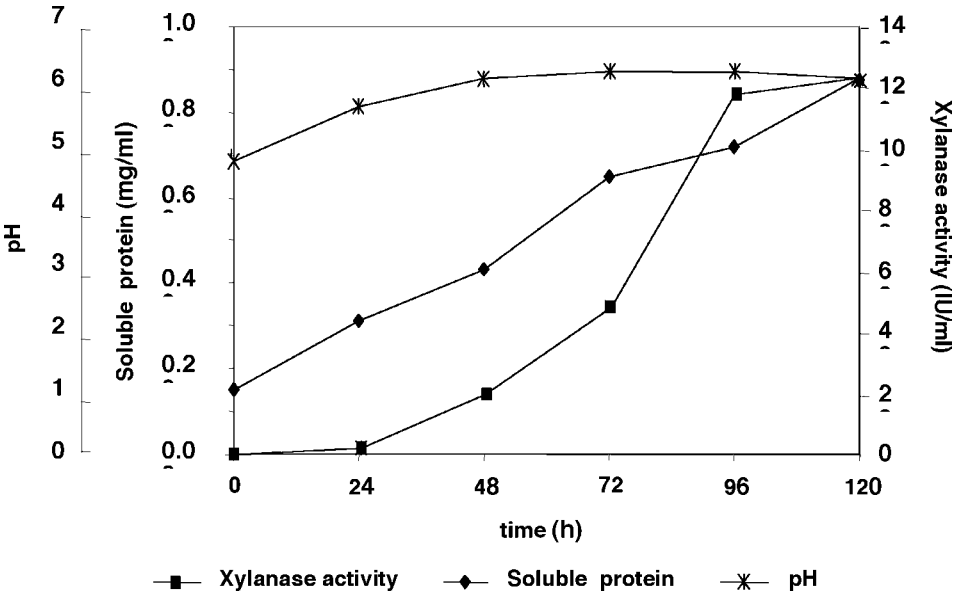

Fig. 5. Xylanase production, pH variation, and soluble protein in Bioflo fermentation of *T. reesei* Rut C-30 on rice straw. Initial pH = 4.8.

When the best rice straw fermentation obtained in shake flaks (initial pH 4.8) was run in a fermentor, xylanase production was greater and more consistent (123 IU/mL) than in shake flaks, although it was similar by 72 h (Fig. 5). Better culture conditions in the fermentor could explain this behavior, mainly owing to better agitation and an adequate air supply. As a result, the decrease in xylanase production found in shake-flask fermentation by 96 h was not observed in the fermentor. The trends in pH and soluble protein were similar to corresponding values in the initial pH 4.8 shake-flask fermentation.

Xylanases produced in the fermentor were used to hydrolyze untreated and ammonia-treated dwarf elephant grass (Table 1) and compared with the performance of a commercial xylanase. As expected, the treated substrate had greater sugar yields (279–301 mg/g of dry matter [DM]) than the untreated one (89 to 90 mg/g of DM), confirming the efficacy of the ammonia treatment in increasing the susceptibility of lignocellulosics to enzymatic hydrolysis. Susceptible fiber in the untreated material was almost completely hydrolyzed by 6 h of hydrolysis, whereas a 24-h period was not sufficient to hydrolyze the available fiber in the treated material. More important, there were no significant differences ($p < 0.05$) in sugar conversion between commercial enzymes and enzymes produced by *T. reesei* Rut C-30 on rice straw. A sugar yield of 56% of theoretical is considered high for a 24-h hydrolysis period and a very low enzyme loading of 1 IU/g of DM for xylanases and cellulases.

Table 1
Reducing Sugar Yield (mg/g DM) from Enzymatic Hydrolysis of Untreated and Ammonia-Treated Dwarf Elephant Grass with Xylanases Produced by *T. reesei* Rut C-30 on Rice Straw and Multifect XL[a]

Enzymes and substrates	Hydrolysis time (h)			
	0	6	12	24
T. reesei				
Treated	83 ± 3.83 ^{aA}	210 ± 7.95 ^{bA}	244 ± 3.32 ^{cA}	301 ± 4.07 ^{dA}
Untreated	52 ± 6.75 ^{aB}	78 ± 3.19 ^{bB}	94 ± 4.02 ^{bB}	89 ± 6.66 ^{bB}
Multifect XL				
Treated	86 ± 1.34 ^{aA}	199 ± 9.91 ^{bA}	231 ± 16.6 ^{cA}	279 ± 14.75 ^{dA}
Untreated	53 ± 10.48 ^{aB}	78 ± 3.7 ^{aB}	92 ± 4.57 ^{bB}	90 ± 7.24 ^{bB}

[a]Results in rows with different capital letters are significantly different ($p < 0.05$). Results in columns with different lower-case letters are significantly different ($p < 0.05$).

Conclusions

The differences observed in the fermentations are basically owing to the nature of the substrates (lignocellulosic and commercial ones) and to the effect of the initial and the developed pH on fungus growth and enzyme production. Rice straw is an excellent substrate for the production of xylanases. An initial pH of 4.8 is appropriate for the production of xylanases. Xylanases produced by *T. reesei* Rut C-30 are effective for the hydrolysis of ammonia-treated dwarf elephant grass.

Acknowledgments

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