

Evaluation of hollocelulase production by *Lentinula edodes* (Berk.) Pegler during the submerged fermentation growth using RSM

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(With 7 figures)

Abstract

The cellulase proteins have a great importance in the enzymatic hydrolysis of woody biomass. Despite of costs being a major concern, it has been a stimulus to study basidiomycetes biochemical properties which degrade lignocellulosic material and have prompted the processes' study for obtaining cellulolytic enzymes in fungi. The objective of this research was to evaluate the effects of the initial nitrogen content on (ammonium sulfate) and on sugar cane bagasse, which hereby, acts as an inducer of hydrolytic enzymes to produce cellulases and xylanases, using three *Lentinula edodes* (Berk.) Pegler strains as a transformation agent. A factorial design with 2² replications in the central point was conducted, varying concentrations of ammonium sulfate and sugar cane bagasse. The submerged cultures carried out in synthetic culture medium and incubated at 25°C for 7 days on an orbital shaker at 150 rpm. The total protein and cellulase activity as endoglucanase, exoglucanase and β -glucosidase and the xylanase was also determined. The results showed that the production of hydrolytic enzymes was stimulated by the presence of high concentrations of sugar cane bagasse (30g/L), characterizing it as an inducer due to the demonstrated proportional relationship. Thus, ammonium sulfate acted as a reducing agent in the synthesis of enzymes, being the low concentrations (0.1g/L) indicated for the enzyme production system under study. Among the studied strains, the EF52 showed higher activity for xylanase, endoglucanases, β -glucosidase and also protein.

Keywords: basidiomycetes, sugar cane bagasse, ammonium sulfate, hydrolytic enzymes, proteins.

Avaliação da produção de enzimas holocelulolíticas por *Lentinula edodes* (Berk.) Pegler durante o crescimento em fermentação submersa usando MSR

Resumo

As celulasas são proteínas de grande importância na hidrólise enzimática de biomassa florestal. No entanto, seu custo elevado tem estimulado o estudo de processos de obtenção de enzimas celolíticas por fungos filamentosos, tais como os basidiomicetos que apresentam propriedades bioquímicas para degradação de material lignocelulósico. O objetivo deste trabalho foi avaliar os efeitos do teor inicial de nitrogênio (sulfato de amônia) e de um indutor de enzimas hidrolíticas (bagaço de cana de açúcar) na produção de xilanases e celulasas utilizando três isolados de *Lentinula edodes* (Berk.) Pegler como agente de transformação. Foi realizado um planejamento fatorial 2² com repetição no ponto central, variando as concentrações de sulfato de amônia e bagaço de cana de açúcar. O cultivo submerso realizado em meio de cultivo sintético e incubado a 25°C por 7 dias em agitador orbital a 150 rpm. Foram determinados o teor de proteínas totais e a atividade de celulase como: endoglucanase, exoglucanase e β -glucosidase e ainda xilanase. Os resultados demonstraram que a produção das enzimas hidrolíticas foi estimulada pela presença de alta concentração de bagaço de cana (30g/L), caracterizando-o como agente indutor devido à relação de proporcionalidade demonstrada. Por sua vez, o sulfato de amônia atuou como redutor da síntese de enzimas, sendo as baixas concentrações (0,1g/L) indicadas para o sistema de produção das enzimas em estudo. Quanto às linhagens, a EF52 mostrou maior atividade para xilanase, endoglucanases, β -glucosidase e proteínas.

Palavras-chave: basidiomicetos, bagaço de cana, sulfato de amônia, enzimas hidrolíticas, proteínas.

1. Introduction

Notwithstanding the growing concern regarding the political issues between countries such as the decrease of oil reserves, increasing demand and dependence on fossil fuels, climate change and environmental pollution, research on renewable energy sources to reduce such problems have become a matter of widespread attention (De Oliveira et al., 2005).

Global production of biofuels is based on the use of raw sugary materials (sugar cane in Brazil) or starch (corn in the United States) (Jardine et al., 2009). However, the first generation ethanol suffers from factors as competition for food crops. Conversely, its large-scale development raises concerns about their direct and indirect effects on land use (Gnansounou, 2010).

The use of lignocellulosic biomass waste stemmed from agriculture and forestry is a viable alternative which can bring savings to fuel production. Some of the agro-industrial residues have a higher proportion of cellulose up to 75% in its content, which justifies the use of this material in the production of cellulases (Adsul et al., 2004) or metabolites in bioconversion of high value, such as ethanol (Tao et al., 2010).

Therefore, new techniques like the submerged culture, a system capable of generating a variety of metabolites and in which the filamentous fungi have great importance (Gomes et al., 2008; Papagianni, 2004). The submerged cultivation in relation to the solid substrate fermentation has the advantage of being more homogeneous system (Mtui, 2012; Aguilar et al., 2004), allowing improved streamlining and standardization of the process (Hölker and Lenz, 2005).

The bagasse usage from sugar cane crops in the submerged culture produces metabolites suchlike cellulases, lignases, xylanases, ethanol, citric acid, lactic acid and others. Furthermore, sugar cane bagasse may be used as a support to the development of microorganisms allowing aerobic conditions for their homogeneous growth (Song and Wei, 2010; Pandey et al., 2000).

Fungi comprehend a heterogeneous group of heterotrophic microorganisms that act as saprobes or parasites or, less frequently, as symbionts (Gomes et al., 2008). *Lentinula edodes* belongs to a group of fungi, white rot type, which are known for efficient capacity of degradation of lignocellulosic materials in nature, especially wood. System degradation of lignin by these fungi has potential applications in biobleaching in the cellulose and paper industries, biodegradation of pollutants and in the production of biofuels. Among the extracellular enzymes produced by the *L. edodes* enzyme system are the endoglucanases, exoglucanases, β -glucosidases and xylanases. Their activities being related to and dependent on substrate composition and environmental factors (Elisashvili et al., 2008).

Cellulase application is one of the most effective methods for improving the yield of sugar from lignocellulose in obtaining bioethanol. For the time being, its wider use is still economically impracticable nowadays. It is therefore

imperative to develop studies for producing large quantities of cellulose at lower costs.

This study aimed to evaluate the effects of the initial content of nitrogen (ammonium sulfate) and an inducer of hydrolytic enzymes (sugar cane bagasse) to produce holocelulases by *Lentinula edodes* as agents of change.

2. Material and Methods

2.1. Microorganism

The microorganism used in the experiment was the fungus basidiomycete *Lentinula edodes* (EF 49, 50 an 52 isolates) Collection of Work Technology Laboratory Non-timber Products from the Brazilian Agricultural Research - National Research Center for Forestry - EMBRAPA FORESTS (Colombo, PR, Brazil). The experiment was conducted at the Laboratory of Biochemical Engineering, at the Regional University of Blumenau, SC, Brazil, where the isolates were cultivated and kept in Petri dishes containing medium Potato Dextrose Agar (PDA) for seven days in an environmental chamber at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the absence of light and after growth stored at 4°C .

2.2 Enzyme production

The enzymes production experiments were carried using submerged fermentation in the synthetic medium as proposed by Suárez et al. (2005): 0.68g/L of potassium hydrogenphosphate (KH_2PO_4), 0.87g/L potassium phosphate (K_2HPO_4), 0.2g/L magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 0.2g/L calcium chloride (CaCl_2) $2 \times 10^{-3}\text{g/L}$ of zinc sulfate (ZnSO_4) $2 \times 10^{-3}\text{g/L}$ of ferrous sulfate (FeSO_4), $2 \times 10^{-3}\text{g/L}$ magnesium sulfate (MnSO_4) adjusted to pH 6.0.

For studying the effect of industrial residues use as carbon source in the induction of protein production, xylanases and cellulases isolated by *L. edodes*, the sugar cane bagasse, from local companies was used. The processing of the pulp was carried out following the methodology described by Souza et al. (2005), in which the pulp was washed several times with distilled water, dried at 60°C for 40 hours and ground in a knife mill into particle size of 2 mm.

To the synthetic medium was added a source of nitrogen (ammonium sulfate) and a carbon source (sugar cane bagasse), through a 2^2 factorial experimental design with 3 replicates at the central point, as shown in Table 1. As a variable of the response, the amount of protein and activity of xylanase, endoglucanase, exoglucanase and β -glucosidase, was assessed by response surface methodology (RSM).

Fermentations were performed in 250 ml Erlenmeyer flasks containing 150 ml of culture medium. The vials were closed with cotton wool plug wrapped with gauze and autoclaved at 121°C for 15 min. The culture medium with five pellets of *L. edodes* remained in shaker at 150rpm for 7 days at 25°C .

2.3 Enzymatic activities

Extraction of enzyme complex was performed by vacuum filtration. The extracts were centrifuged and stored at 4°C . The xylanase activity was determined by the amount

of reducing sugars released from xylan “birchwood” as described by Bailey et al. (1992). The enzymatic assay was carried out using 0.9 mL of 1% xylan and 0.1 mL of enzyme extract, after 5 minutes of reaction the reducing sugars were measured by the method of 3,5 dinitrosalicylic (DNS) (Miller, 1959).

Endoglucanase and the exoglucanase activities were determined according to the method described by Tanaka et al. (1981). This method is to conduct the hydrolysis of a 0.44% solution of carboxymethylcellulose in sodium acetate buffer 0.05 M pH 5.0 activity to the fraction of carboxymethylcellulase and a 1.1% suspension of the same buffer, microcrystalline cellulose (Avicel) to the fraction avicelase. The reaction was initiated by the addition of 0.9 ml of enzyme extract in 1 ml solution of Avicel or 1 ml of CMC solution for exoglucanase and endoglucanase, respectively. The amount of reducing sugars was determined by the DNS method (Miller, 1959).

The activity of β -glucosidase or cellobiose was determined according to Wood and Garcia-Campayo (1990), using 1 mL of cellobiose solution of 0.53% (diluted in sodium acetate buffer pH 5.0) and 1 ml of the enzyme extract and incubated at 50°C for 30 minutes. The reaction was stopped by immersing the tubes in boiling water for 5 minutes. After the transfer to a cold water bath, the produced glucose was determined using the kit based on the reaction of glucose oxidase-peroxidase by the GOD-POD method.

Protein concentration of the samples was determined through the proposed method by Bradford (1976). To each 300 mL of enzyme extract was added 3 mL of Bradford reagent left to react for 5 minutes at room temperature. As a result, a reading in spectrophotometer at 595 nm was done. The standard curve was made with solutions at concentrations of bovine albumin 50-200 mg/L. The concentration of total protein was expressed in mg/L.

Table 1. Matrix of the experiment. Assays 1 to 9 with repetition of the central point (5.2 and 5.3). Where different concentrations were assessed from -1 nitrogen source (0.1g/L) 0 (2.5g/L) and +1 (5.0g/L), with different concentrations of the carbon source -1 (5.0g/L) 0 (15g/L) +1 (30g/L).

Trials	Sulfate of ammonia (Nitrogen Source)	Sugar cane bagasse (Carbon source)
1	-1	-1
2	-1	0
3	-1	+1
4	0	-1
5.1	0	0
5.2	0	0
5.3	0	0
6	0	+1
7	+1	-1
8	+1	0
9	+1	+1

For all enzyme tests, one enzyme activity unit (U) was defined as the amount of enzyme capable of liberating 1 μ mol of reducing sugars (glucose or xylose) per minute at 50°C and the enzyme activity expressed in UIL^{-1} ($\text{U} = \mu\text{mol mL}^{-1} \text{min}^{-1}$). The significance level for studies of protein production and the conditions study of enzyme extraction showed a 95% confidence. For the models validation, the analysis of variance was used, verifying the coefficient of determination (R^2).

3. Results

The relationship between the variables for the enzymes activity was analyzed using the Response Surface Methodology (RSM), which is a collection of useful mathematical and statistical techniques for the modeling and analysis of problems in which a response of interest is influenced by several variables.

In this study the establishment of optimal conditions for enzyme production was performed as varying the concentrations of carbon source (sugarcane bagasse) and nitrogen (ammonium sulfate) in the submerged medium.

The best exploratory model equations for the activity of enzymes are presented in Table 2, along with the coefficients of determination (R^2). Statistically significant effects at the 95% confidence level, where the response was the enzyme activity and proteins production. CB was cane bagasse (w/v) and AS was ammonium sulfate (w/v).

The coefficient of determination was used as a measure of how well the regression equation fits the data. It is the R square, the correlation coefficient that provides the degree of correlation between the dependent variable, Y , and the independent variable X .

Proteins coefficient to EF49 in this study ($R^2 = 0.95$) was satisfactory. To EF50 and EF52 a correlation coefficient of 0.78 was shown. This value indicated that 22% of the regression variance does not depend on the tested variables. The exoglucanase response had similar correlation coefficient ranging between 0.57 to 0.63 to all strains.

The R^2 for the strains allows the response surface plots obtainment (Figures 1, 2, 3, 4 and 5).

The endoglucanase was expressed by strain EF52 concentrations up to 1 UIL^{-1} production of enzyme. EF50 0.3 UIL^{-1} , followed by 0.24 UIL^{-1} strain EF49. That shows a maximum quantities of sugar cane bagasse as the ammonium sulfate are required in order to occur an increased enzyme production (Figure 1).

The diagram of the response surface (Figure 2) indicates that exodoglucanase is an independent variables function (sugarcane bagasse and ammonium sulfate concentration). The enzyme exoglucanase showed the highest value among the strains. The strain EF49 showed the maximum cellulase activity, 9 UIL^{-1} (Figure 2). It is possible to verify the existence of an optimal region for the enzyme activity which is a combination band. To exoglucanase was verified that lesser sugarcane bagasse and smaller the amount of nitrogen source, the higher was the enzyme production.

Table 2. Mathematical models and coefficients of determination (R²) obtained for the enzyme activities as a function of sugarcane bagasse and ammonium sulfate concentration.

Model of enzyme activities and proteins concentration	R ²
EF 49 Xylanase = 0.3109+0.0021*CB-0.0000*CB^2+0.0913*AS-0.0163*AS^2+0	0,7387
EF 49 Exoglucanase = 3.7058+0.0134*CB-0.0003*CB^2+0.0935*AS+0.0017*AS^2+0	0,6346
EF 49 Endoglucanase = 0.0180-0.0120*CB+0.0004*CB^2+0.1285*AS-0.0217*AS^2+0	0,7206
EF 49 Proteínas = 9.3013-0.4418*CB+0.0037*CB^2+18.0507*AS-2.2632*AS^2+0	0,9583
EF 50 Xylanase = 3.9115+0.0154*CB+0.0000*CB^2+0.2328*AS-0.0429*AS^2+0	0,8458
EF 50 Exoglucanase = 3.9115+0.0154*CB+0.0000*CB^2+0.2328*AS-0.0429*AS^2+0	0,5785
EF 50 Endoglucanase = 0.2017-0.0101*CB+0.0006*CB^2-0.1283*AS+0.0154*AS^2+0	0,7989
EF 50 Proteínas = 0.2017-0.0101*CB+0.0006*CB^2-0.1283*AS+0.0154*AS^2+0	0,7948
EF 52 Xylanase = 0.2820+0.0009*CB+0.0001*CB^2+0.0210*AS-0.0025*AS^2+0	0,9656
EF 52 Exoglucanase = 3.9115+0.0154*CB+0.0000*CB^2+0.2328*AS-0.0429*AS^2+0	0,6287
EF 52 Endoglucanase = 0.000-0.0038*CB+0.0002*CB^2-0.0069*AS+0.0023*AS^2+0	0,9418
EF 52 Proteínas = 13.8754-0.1759*CB+0.0160*CB^2+3.1706*AS-0.5372*AS^2+0	0,7893

*CB= Cane bagasse; AS= Ammonium sulfate.

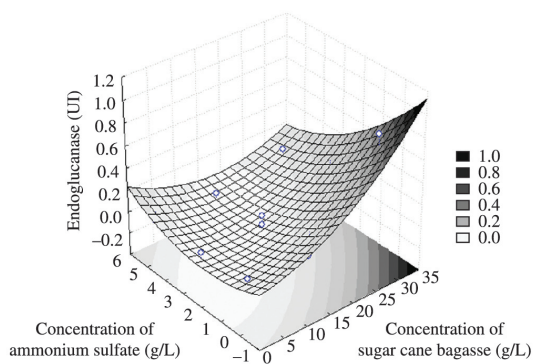


Figure 1. Response surface for the effects of different concentrations of carbon and nitrogen in the production of endoglucanases on EF52 strain of *L. edodes*, where the maximum of the production was 1.0UL⁻¹

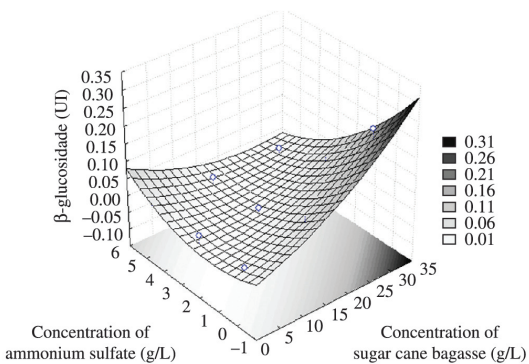


Figure 3. Response surface for the effects of different concentrations of carbon (sugarcane bagasse) and nitrogen (ammonium sulfate) in the production of β -Glucosidase on EF52 strain of *L. edodes*, where the production was 0.31UL⁻¹.

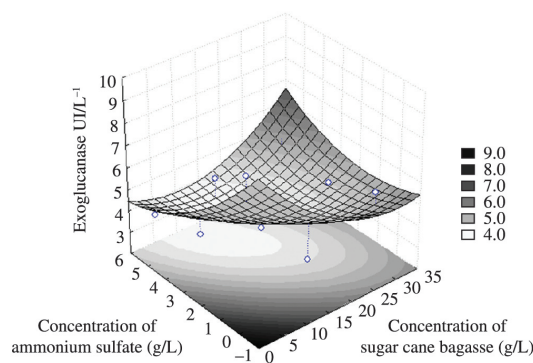


Figure 2. Response surface for the effects of different concentrations of carbon (sugarcane bagasse) and nitrogen (ammonium sulfate) in the production of exoglucanases on EF49 strain of *L. edodes*, where the maximum of the production was 9.0UL⁻¹.

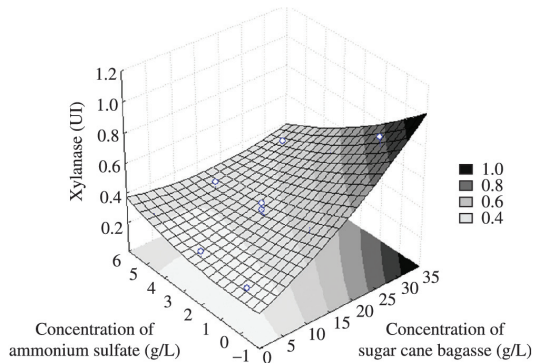


Figure 4. Response surface for the effects of different concentrations of carbon (sugarcane bagasse) and nitrogen (ammonium sulfate) in the production of xylanase on EF52 strain of *L. edodes*, where the maximum production was 1.0UL⁻¹.

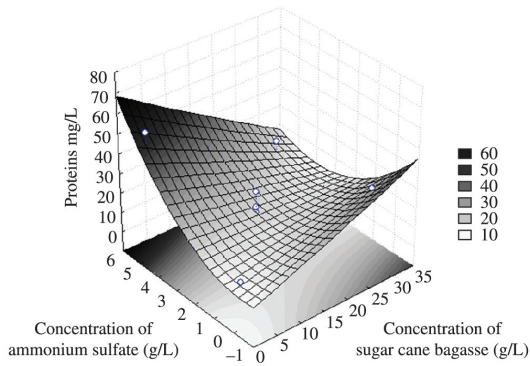


Figure 5. Response surface for the effects of different concentrations of carbon (sugarcane bagasse) and nitrogen (ammonium sulfate) in the production of proteins on EF52 strain of *L. edodes*, where the maximum production was 60mg/L.

Also, the EF52 strain produced 4.8 UI L⁻¹ and the EF50 strain produced 4.4 UI L⁻¹.

The enzyme β -glucosidase was produced only on the EF52 strain with 0.31 UI L⁻¹. There was no enzyme production on EF49 and EF50 strains (Figure 3). The EF52 strain (Figure 4), is also noted in the production of 1UI L⁻¹ xylanase followed by 0.48UI L⁻¹ to EF49 strain and 0.45 UI L⁻¹ to EF50 strain.

EF52 strain produced proteins with values up to 60mg/L. Bulk of ammonium sulfate and minor amounts of sugar cane bagasse generated best results in the production of proteins (Figure 5). Also the EF50 strain produced 40mg/L and the EF49 strain produced 30mg/L.

The Pareto chart for the enzyme production by the strains showed that the linear variable sugarcane bagasse (CB) has a positive effect on the enzyme production, but ammonium sulfate (AS) had a negative effect. The interaction between strains was also significant (Figure 6).

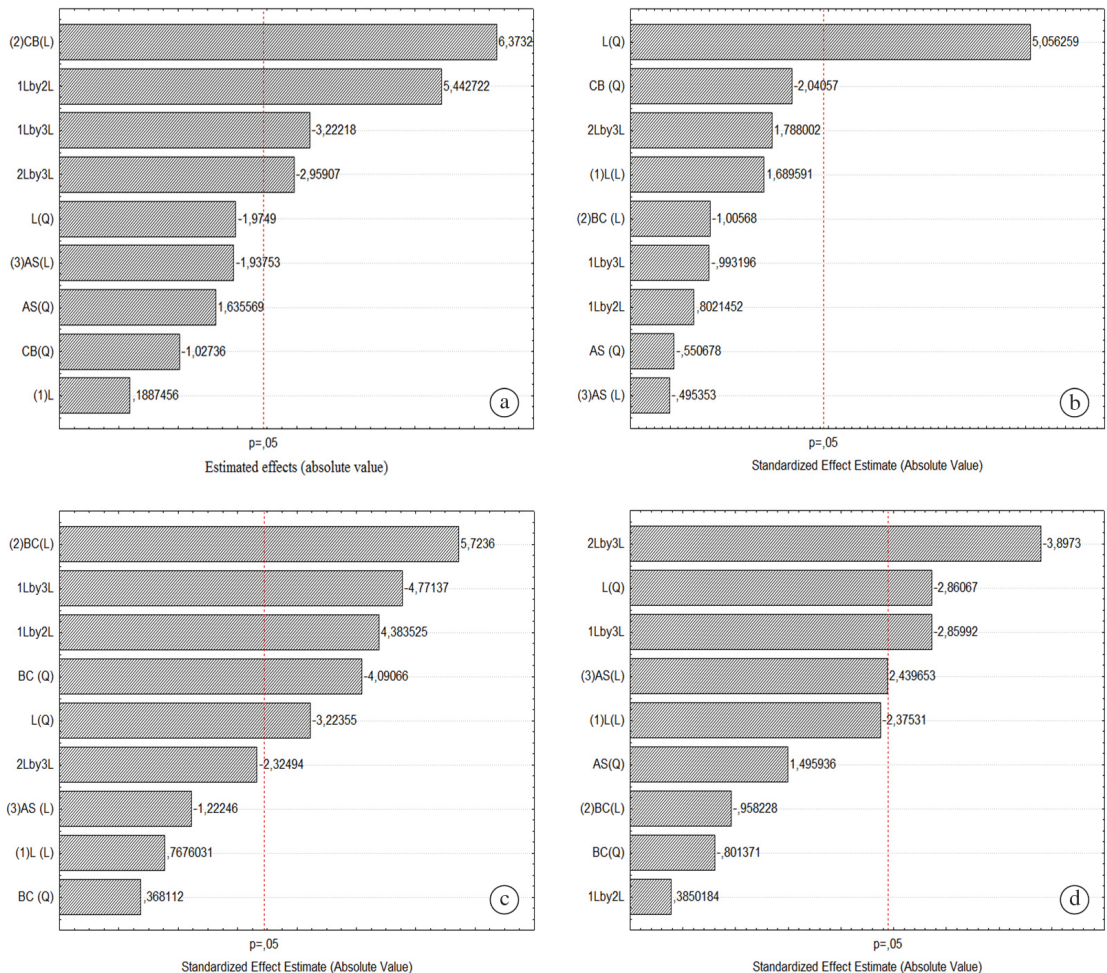


Figure 6. Pareto chart of the factorial design for the influence of the independent variable sugarcane bagasse and ammonium sulfate on enzyme production by *L. edodes* (a. xylanase; b. endoglucanase; c. exoglucanase; d. proteins)

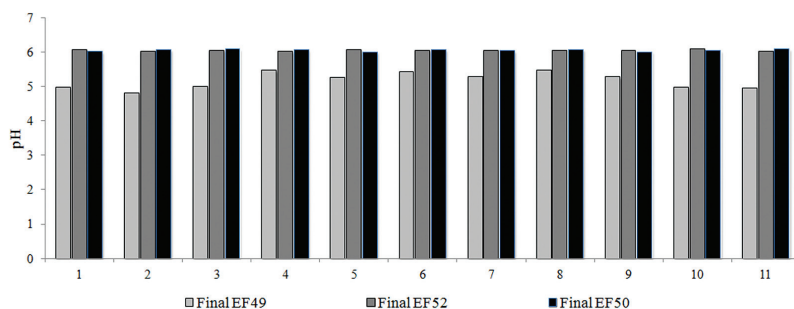


Figure 7. Values of pH the three strains *L. edodes* cultivated for 7 days at shaker with 150rpm at 25°C. The initial pH was 7 for all the strains.

For the xylanase and the exoglucanase (6a, 6c) the concentration of sugar cane bagasse and also the strains were significant. Whereas for the endoglucanase and proteins (6b, 6d), only the strains was significant.

The three strains of *L. edodes* tended to acidification as shown in Figure 7. However, the EF49 strain was the one with reduced pH, reaching values lower than five. Even so, the EF52 and EF50 strains were not lower than pH 7 at seven days of culture.

4. Discussion

The experimental design is an important tool to analyze the enzyme production under different culture conditions, allowing the independent variables interaction analysis. In a study by Juhász et al. (2005), *Trichoderma reesei* and different use of waste as a carbon source, such as cellulose delignified, in treated willow, conifer and corn cob showed determinants that are sources for the production of β -glucosidase. However, the production of β -glucosidase by the genus *Trichoderma* is not as high as to *Aspergillus phoenicis*, which enhances the ability of this enzyme as a producer and the use of mixed cultures for the production of enzymes (Duff et al., 1986; Wen et al., 2005). As the genus *Trichoderma*, the species *L. edodes* does not seem to be a good producer of β -glucosidase enzyme.

Mendoza et al. (2009) in their study had the highest xylanolytic activity (0.095 IU/mL) and cellulolytic (endoglucanases: CMCase 0.326 IU/mL, FPase 0.0635 IU/mL) when the *T. hanzianum* was cultured in medium with sugarcane bagasse. Considering the proximity of the polymers cellulose and hemicellulose in plant wall, it was not surprising that xylanolytic and cellulolytic activities of *T. hanzianum* were related.

Thus, according to Stricker et al. (2008), the nature of these hydrolases work synergistically to achieve complete degradation of biopolymers present on substrates, which does not corroborate with the results, where the amount of one of cellulases produced two times more than the xylanase.

For Jatinder et al. (2006), ammonium sulfate had also a positive effect on production of cellulases and xylanases to the fungus *S. termophilum*, however, in excess it inhibited the production of the same.

Garcia-Kirchner et al. (2002) cultivating *Penicillium* sp and *A. terreus* using liquid medium with sugarcane bagasse as carbon source and corn steep liquor as a nitrogen source found similar results in the production of enzymes.

Few studies have demonstrated the effects of different nitrogen sources on the production of fungal cellulases. For Irfan et al. (2012) ammonium sulfate showed less enzyme production by fungi as compared to organic nitrogen sources like peptone, but is as good as yeast extract and better than casein.

In some studies it was observed that there were differences in enzyme activity among isolates of the same genus of *Lentinula*, which shows that not all members of the same genus have the same potential for the production of enzymes (Philippoussis et al., 2011).

Data obtained by Höfling et al. (2001) suggest that different nutritional compositions in the media, expressed by the electrophoretic profiles determine the expression of different proteins derived from alternative metabolic mechanisms.

Although, the comparison of these results to those from other studies is limited due to the wide variety of microorganisms used, as well as the fermentation conditions. All these factors cause a wide range of values in the literature.

The results indicated that for the cellulases and xylanases production, the use of ammonium sulfate as sole nitrogen source had a positive effect on the activity of the endoglucanase and proteins, under linear, but inhibited the production exoglucanases.

The production of hydrolytic enzymes was stimulated by the presence of higher concentration of the source of bagasse, characterizing it as an inducer. In turn, the nitrogen source use, ammonium sulfate, acted as a reducing agent in the synthesis of enzymes, and the lowest concentrations indicated for the cellulases and xylanases system production. Consequently, strains of *L. edodes* shown to produce different amounts of enzymes.

The pH value is an important variable for enzyme activity (Arantes et al., 2011). The tendency to acid pH values during the culture in liquid medium in order to produce fungal cellulases filamentous have been described in the literature (Chen and Wayman, 1991). In some

studies it was found that there was in the culture media, a tendency to acidification during the first 48 hours, but in most of them, the pH stabilized or increased gradually. The low values are probably due to aerobic microbial activity (Antonio and Bianchini Junior, 2003). For further studies there must be tests using buffer or other residue that can simultaneously maintain the pH stabilization.

Besides the variety of enzyme production as well as the amount its production, in this study the strain which was determined as the largest number of enzymes was the EF52.

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