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PRODUÇÃO DE CELULASES POR *Gelatoporia subvermispora* PARA HIDRÓLISE DE MATERIAL CELULÓSICO

DISSERTAÇÃO DE MESTRADO

Cristiane Bianchi Loureiro dos Reis

Santa Maria, RS, Brasil

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PRODUÇÃO DE CELULASES POR *Gelatoporia subvermispora* PARA HIDRÓLISE DE MATERIAL CELULÓSICO

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Orientador: Prof. Dr. Rodrigo Josemar Seminoti Jacques

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elaborada por
Cristiane Bianchi Loureiro dos Reis

como requisito parcial para a obtenção do grau de
Mestre em Ciência do Solo

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Santa Maria, 16 de Abril de 2014.

À minha mãe Jucélia e ao meu pai Luiz Carlos
Ao meu amado Aldoir Junior
À minha sogra Gladis (in memoriam)

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciência do Solo
Universidade Federal de Santa Maria

PRODUÇÃO DE CELULASES POR *Gelatoporia subvermispora* PARA HIDRÓLISE DE MATERIAL CELULÓSICO

AUTORA: CRISTIANE B. LOUREIRO DOS REIS
ORIENTADOR: RODRIGO J. S. JACQUES
Data e Local da Defesa: Santa Maria, 16 de abril de 2014.

Diversos microrganismos são capazes de produzir enzimas responsáveis pela conversão da biomassa em etanol, tais como as celulases, porém, estudos sobre a diversidade e aplicação biotecnológica dos microrganismos do bioma Pampa ainda são escassos. Para tentar reduzir essa lacuna de conhecimento, o fungo *Gelatoporia subvermispora* foi isolado do bioma Pampa, resultando na primeira ocorrência dessa espécie para a América Latina. Comercialmente, as celulases são produzidas através de fermentação submersa. Entretanto, fungos filamentosos que são considerados bons produtores de celulases apresentam melhores resultados em fermentação em estado sólido. Para avaliar o potencial biotecnológico do fungo *G. subvermispora* de produzir celulases os resíduos palha de arroz, lodo de esgoto e bagaço de cana foram utilizados como substrato. Os maiores valores de atividade celulolítica total determinados pelo método de papel filtro (PF) foram obtidos utilizando bagaço de cana como substrato, apresentando 3.82 $FPU.g^{-1}$. O lodo de esgoto foi um excelente meio para a produção de xilanases e exo-celulases, atingindo valores máximos de 227.97 $U.g^{-1}$ e 134.25 $U.g^{-1}$, respectivamente. A atividade da endo-celulase foi similar nos substratos testados, como verificado na condição 11 para a palha de arroz (40.75 $U.g^{-1}$) e 14 para o lodo de esgoto (35.32 $U.g^{-1}$). As enzimas celulolíticas produzidas pelo fungo *Gelatoporia subvermispora* foram aplicadas na hidrólise do bagaço de cana por meio de sonicação indireta. Os melhores resultados foram obtidos utilizando o lodo de esgoto como substrato para a produção das enzimas. Os rendimentos médios de hidrólise obtidos através da produção de enzimas utilizando lodo de esgoto, bagaço de cana e palha de arroz como substrato foram 72.8, 58.7 e 51.2 $g.kg^{-1}$, respectivamente. Com relação à utilização do ultrassom para as reações enzimáticas, a amplitude apresentou um efeito negativo no rendimento, enquanto o fator pulso foi benéfico para as reações.

Palavras-chave: Fungos filamentosos. Resíduos lignocelulósicos. Fermentação em estado sólido. Enzimas.

ABSTRACT

Master dissertation
Graduate Program in Soil Science
Federal university of Santa Maria

PRODUCTION OF CELLULASES BY *Gelatoporia subvermispora* TO CELLULOSIC MATERIAL HYDROLYSIS

AUTHOR: CRISTIANE B. LOUREIRO DOS REIS
ADVISOR: RODRIGO J. S. JACQUES
Date and Local of the Defense: Santa Maria, april 16th 2014.

Several microorganisms are able of producing enzymes responsible for the conversion of biomass to ethanol, such as cellulases. However, studies about diversity and biotechnological application of microorganisms from Pampa biome are still scarce. To try to reduce this knowledge lack, the *Gelatoporia subvermispora* fungus was isolated from the Pampa biome, resulting in the first occurrence of this specie in Latin America. Commercially, cellulases are produced by submerged fermentation. However, filamentous fungi that are considered good producers of cellulases show better results in solid-state fermentation, mainly by the similarity between the solid medium and the natural habitat of these microorganisms. In order to evaluate the biotechnological potential of the fungi *G. subvermispora* for cellulases production, rice straw, sewage sludge and sugar cane bagasse were used as substrate. The highest values for the total cellulolytic activity by the method of filter paper (FP) were obtained using sugarcane bagasse as substrate, reaching the value of 3.82 $FPU.g^{-1}$. The sewage sludge was an excellent medium for the production of xylanase and exo-cellulases, reaching peak activity of 227.97 $U.g^{-1}$ and 134.25 $U.g^{-1}$, respectively. The endo-cellulase activity was similar in almost whole substrates tested, as showed in the runs 11 for rice straw (40.75 $U.g^{-1}$) and 14 for sewage sludge (35.32 $U.g^{-1}$). Subsequently, cellulolytic enzymes from fungal *Gelatoporia subvermispora* produced by solid-state fermentation using different substrates were applied to hydrolyze non-treated sugarcane bagasse using indirect sonication. The best results were obtained using sewage sludge as substrate for enzyme production. The mean yields obtained with enzymes produced using sewage sludge, sugarcane bagasse and rice straw as substrates were 72.8, 58.7 and 51.2 $g.kg^{-1}$, respectively. Regarding the use of ultrasound to carry out enzymatic reactions, the oscillation amplitude presented a negative effect on yield, whereas pulse factor showed to be benefic for the reactions.

Keywords: Filamentous fungi. Lignocellulosic residues. Solid-state fermentation. Enzymes.

SUMÁRIO

INTRODUÇÃO

A demanda energética mundial tem incentivado pesquisas de fontes renováveis de energia, dentre elas o etanol. No Brasil, apesar da grande produção de etanol a partir da cana-de-açúcar, a produção de álcool derivado da biomassa lignocelulósica também é uma alternativa viável e sustentável (MUSSATTO et al., 2010). Por esse motivo, os materiais de origem lignocelulósica provenientes de atividades agrícolas, como bagaço de cana-de-açúcar, palha, madeira, restos de culturas de grãos e frutíferas, assim como a fração orgânica dos resíduos sólidos urbanos têm sido utilizados visando o emprego em processos para renovação energética. Tais materiais são ricos em sacarídeos, alguns muito complexos, sendo a conversão dessas moléculas vista como uma alternativa na substituição de combustíveis fósseis (MAEDA et al., 2013).

Os materiais lignocelulósicos são compostos por celulose, hemicelulose e lignina. Basicamente, a celulose forma um esqueleto que é composto por substâncias estruturais (hemicelulose) e envoltórias (lignina) (FENGEL e WEGENER, 1989; PÉREZ et al., 2002). A conversão desses materiais em etanol pode ser realizada através de um sistema de enzimas capazes de hidrolisar a celulose da biomassa em glicose. Tal processo requer a ação de três grupos de celulases, as quais segundo Lynd et al. (2002), atuam de forma sinérgica e diferentemente conforme o organismo e o substrato. As endoglucanases (E.C. 3.2.1.4) são capazes de hidrolisar os polímeros de celulose internamente; as exoglucanases ou celobiohidrolases (E.C. 3.2.1.91) são responsáveis pela liberação de celobiose ou celo-oligossacarídeos, enquanto as β–glucosidases (E.C. 3.2.1.21) hidrolisam a celobiose, e outros celo-oligossacarídeos curtos, à glicose (MAEDA et al., 2011; DELABONA et al., 2013; GOTTSCHALK et al., 2010).

O principal impedimento para a produção de etanol lignocelulósico, também conhecido como etanol de segunda geração, deve-se a alta dos preços da celulase no mercado, podendo representar cerca de 50% do custo de produção. Desta forma, o desenvolvimento de novas tecnológicas e equipamentos mais eficientes para a produção de celulases é crucial para superar este problema e tornar a produção de etanol lignocelulósico economicamente viável (ZHANG et al., 2006).

Comercialmente, a maioria das celulases são produzidas por meio de fermentação submersa (SmF) devido aos fatores da fermentação serem mais facilmente controlados e mantidos (TOLAN e FOODY, 1999). No entanto, os fungos filamentosos, considerados bons

produtores de celulases e xilanases, apresentam melhor desempenho através da fermentação em estado sólido (SSF), devido ao fato desse método apresentar maior similaridade com o habitat natural dos fungos do que em fermentação submersa (MITCHELL et al., 2006). Além disso, a fermentação em estado sólido apresenta diversas vantagens em relação à fermentação submersa, como maior rendimento de produção, menor geração de resíduos e economia de energia no processo de fermentação e purificação da enzima. Desse modo, resíduos agroindustriais podem ser utilizados como substratos para a fermentação em estado sólido, possibilitando utilizar o extrato enzimático para hidrolisar o material celulósico, reduzindo significativamente os custos do processo de produção (ROCKY-SALIMI e HAMIDI-ESFAHANI, 2010; LATIFIAN et al., 2007).

As celulases podem ser produzidas por fungos filamentosos, como já mencionados, mas também por outros microrganismos. Neste contexto, a bioprospecção de microrganismos bons produtores de celulases, ou de celulases melhoradas, tem despertado grande interesse. A América Latina apresenta vários centros de biodiversidade, os quais além de serem pouco explorados, principalmente no que diz respeito aos microrganismos do solo, encontram-se em processo de degradação e consequente perda de biodiversidade. Assim, muitos microrganismos pertencentes a biomas latinos, como o bioma Pampa, ainda são desconhecidos e podem em um futuro próximo serem extintos devido à degradação ambiental. Na tentativa de diminuir essa lacuna de conhecimento, diversos estudos foram realizados no intuito de se obter uma coleção de fungos celulolíticos oriundos do bioma Pampa, localizado no sul da América Latina, dos quais resultou a obtenção da primeira ocorrência do fungo *Gelatoporia subvermispora* para a América Latina (BALDONI, 2012).

Além da busca por microrganismos capazes de produzir celulases, a pesquisa atual tem se preocupado em buscar alternativas para reduzir os custos dos processos envolvidos na produção do etanol lignocelulósico. Dentre as alternativas destacam-se o emprego da mesma biomassa lignocelulósica para a produção de celulases e para o processo de hidrólise e a redução do tempo de fermentação (DELABONA et al., 2012; LEAES et al., 2013). Mais recentemente, o efeito do ultrassom no rendimento da hidrólise do material lignocelulósico tem sido estudado. Vários autores demonstraram que a sacarificação da celulose é aumentada de forma eficiente pelo pré-tratamento com ultrassom (ALVIRA et al., 2010; KARKI et al., 2011; TIAN et al., 2012; PEJIN et al., 2012). O funcionamento do ultrassom é baseado no fenômeno da cavitação, o qual se caracteriza pela formação, crescimento e colapso de bolhas de gás ou vapor (SANTOS JR et al., 2008). Os maiores rendimentos de hidrólise enzimática após pré-tratamento com ultrassons são explicados porque a introdução do ultrassom no

material lignocelulósico aumenta grandemente o transporte de água para a celulose cristalina. Além disso, o impacto mecânico produzido pelo colapso das bolhas fornece um benefício importante ao abrir a superfície dos substratos sólidos para a ação das enzimas (ALVIRA et al., 2011; GOGATE e KABADI, 2009; SZABÓ e CSISZÁR, 2013).

Diante disso, formulou-se a hipótese de que os fungos lignocelulolíticos do solo do bioma Pampa podem produzir celulases através de fermentação em estado sólido, utilizando os resíduos lignocelulolíticos. Portanto, um dos objetivos do estudo foi avaliar as melhores condições para a produção de enzimas celulolíticas pelo fungo *G. subvermispora* (BALDONI, 2012). Para tanto, diferentes resíduos lignocelulósicos de baixo valor e emprego comercial foram utilizados como substrato, tais como o bagaço de cana, a palha de arroz e o lodo de esgoto. Outro objetivo foi avaliar o efeito da aplicação das enzimas celulolíticas produzidas por *Gelatoporia subvermispora* utilizando os mesmos substratos na hidrólise de bagaço de cana através de sonicação indireta.

PRODUCTION OF CELLULOLYTIC ENZYMES USING DIFFERENT SUBSTRATES FROM FUNGAL *Gelatoporia subvermispora* ISOLATED IN LATIN AMERICAN

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Abstract

In this study was optimized the production of cellulolityc enzymes by the fungal *Gelatoporia subvermispora* using the solid-state fermentation. The experiments were conducted in conical flasks. The enzyme activities of filter paper, exo-cellulase, endo-cellulase and xylanase were determined. Different cheap solid agro-lignocellulose wastes were used as substrate including sugarcane bagasse, sewage sludge and rice straw. A Plackett–Burman design (PB) was used to determine the important variables in the fungal enzyme production by solid-state fermentation. The evaluated variables were moisture content of substrate (60 to 80%), inoculums density (1 to 3 mycelium discs of 3 mm), corn steep liquor (0 to 15% v/w), pH (5 to 7) and pepton concentration (5 to 20 wt%). The optimized conditions for production determined in a central composite rotational design (CCRD) using rice straw as substrate were moisture content (40%) and pepton concentration (5wt %), while the better condition for production using sugarcane bagasse or sewage sludge were determined based on the results obtained in previous assays. The highest values for the total cellulolytic activity by the method of filter paper (FP) were obtained using sugarcane bagasse as substrate, reaching the value of 3.82 $FPU.g^{-1}$. The sewage sludge was an excellent medium for the production of xylanase and exo-cellulases, reaching peak activity of 227.97 $U.g^{-1}$ and 134.25 $U.g^{-1}$, respectively. The endo-cellulase activity was similar in almost whole substrates tested, as showed in the runs 11 for rice straw (40.75 $U.g^{-1}$) and 14 for sewage sludge (35.32 $U.g^{-1}$).

Keywords: Pampa biome. Cellulases. Agro-lignocellulose wastes. Solid-state fermentation.

Introduction

The new concept of ethanol (or bioethanol) corresponds to its production utilizing lignocellulosic biomass as low-cost agricultural and forest residues, wood process wastes, and the organic fraction of municipal solid wastes. Where these materials are available, it should be possible to produce ethanol with virtually no additional land requirements or impacts on food and fiber crop productions (Sims et al. 2008).

The conversion of lignocellulosic biomass into fuels can be achieved using enzyme systems acting in order to hydrolyze biomass to glucose. It is well established that hydrolytic efficiency is a result of the synergistic actions of a multicomponent enzymatic systems, containing at least three major groups of enzymes: endo-glucanases (E.C. 3.2.1.4), which hydrolyze the cellulose polymer internally, exposing reducing and non-reducing ends; exo-glucanases or cellobiohydrolases (E.C. 3.2.1.91), which act on the reducing and non-reducing ends, releasing cellobiose and cellooligosaccharides; and β–glucosidases (E.C. 3.2.1.21), which cleaves cellobiose, liberating two molecules of glucose–the end product (Maeda et al. 2011; Delabona et al. 2013; Gottschalk et al. 2010).

Cellulases can be produced and secreted by filamentous fungi and other microorganisms. Accordingly, Latin America has several biodiversity hot spots, which were not studied, particularly with regard to the diversity of soil microorganisms. Nevertheless, many Latinos biomes are under intense process of degradation and loss of biodiversity. So many microorganisms and biotechnological potential are being extinct without at least be known and preserved. To try to reduce this lack of information, collections of cellulolytic fungi were made in Pampa Brazilian biome, located in southern Latin America, which resulted in the first report of the occurrence of *Gelatoporia subvermispora* in Latin America (Baldoni 2012), whose potential cellulolytic was previously unknown. This way, the use of

cellulase-producing microorganisms and the development of new technological routes for cellulases production remains a strategic issue to be considered during the development of a sustainable process for ethanol production from biomass. Commercially, most of cellulases enzymes were produced through submerged fermentation (SmF) due to easier controlled and maintained fermentations factors (Tolan and Foody 1999). However, the filamentous fungi which considered as strong cellulases secreting strains perform better using solid-state fermentation (SSF) since solid medium could simulate fungi natural habitat (Mitchell et al. 2006). Furthermore, SSF was more advantageous since it has greater volumetric productivities, higher product stability, low contamination risk and lower instrumental costs (Mitchell et al. 2006).

Therefore, the aim of this study was to evaluate production of cellulolytic enzymes by the fungal *G. subvermispora* (Pilát) Niemelä (1985) isolated first time in Latin American. Different cheap solid agro-lignocellulose wastes were used including sugarcane bagasse, sewage sludge and rice straw. Optimized conditions were also encountered for cellulase production by *G. subvermispora*.

Material and methods

Microorganism

Gelatoporia subvermispora (Pilát) Niemelä (1985) was first time isolated from Brazilian Pampa Biome in a pine forest by Baldoni (2012). Stock cultures of *G. subvermispora* were propagated on potato dextrose agar (PDA) using Petri plates at 28°C for five days.

Solid substrate

Sugarcane bagasse was obtained in a micro distillery of bioethanol production and rice straw was obtained from rice processing industry. In the laboratory, the residues were dried at 60°C during 24 hours, grounded in a cutting mill and sieved with final particle size of 8 mesh. The solid substrate for enzyme production was composed by sugarcane bagasse or rice straw as main carbon source, supplemented with pepton and corn steep liquor.

The collection of the activated sludge occurred in November 2012 in a residential treatment station sludge, in the drying beds with longer desague (approximately 90 days) in order to collect more stabilized sludge. After the sewage sludge was dried in an oven with forced air circulation (Brand New Ethics 420 model - 5D) at 40°C for seven days. The dry material was homogenized in mill ground (Marconi brand) and sieved in 2 mesh.

Production of cellulolytic enzymes and their assays

A Plackett–Burman design (PB) was used to determine the important variables in the fungal enzyme production by solid-state fermentation. The evaluated variables were moisture content of substrate (60 to 80%), inoculums density (1 to 3 mycelium discs of 3 mm), corn steep liquor (0 to 15% v/w), pH (5 to 7) and pepton concentration (5 to 20 wt%). A PB design composed of twelve runs plus three central points was accomplished for each substrate (sugarcane bagasse, rice straw and sewage sludge). Table 1 presents the investigated range for each variable of the three PB designs.

The fermentations were carried out in conical flasks (500 mL) containing 10 g of solid substrate. Afterwards, the solid substrate was supplemented and the moisture content adjusted

at specified level. Each flask was covered with hydrophobic cotton and autoclaved at 121°C for 20 min. After cooling, each flask was inoculated with mycelium discs (3 mm diameter) and incubated for 120 h at 28ºC for five days.

The condition of run 7 and 9 of PB design were utilized to available the cellulolytic enzymes using sewage sludge and rice straw as substrate, whereas for sugarcane bagasse the condition of run 12 was used. Based on the analysis of results, a central composite rotational design (CCRD) for two independent variables was conceived to investigate the influence of moisture content (40 to 60%) and peptone concentration (0 to 5wt%) on cellulolytic enzymes production by *G. subvermispora* using rice straw as substrate. The cellulolytic enzymes considered in this work were filter paper activity ($FPU.g^{-1}$), exo-cellulase activity ($U.g^{-1}$), endo-cellulase activity ($U.g^{-1}$) and xylanase activity ($U.g^{-1}$).

Extraction of the cellulolytic enzymes and assays

At the end of fermentation, the cellulolytic enzymes were extracted using 100 mL of 50 Mm sodium acetate buffer (pH 4.8) in an orbital shaker at 120 rpm and 28°C during 1 hour. Afterwards, 30 mL of the enzyme extract was withdrawn for determination of enzymes activities.

Cellulolytic enzymes activities were determined as described by Ghose (1987), with few modifications. The Filter paper activity assay was carried out using 50 mg of filter paper Watmann n°1, 1 mL of diluted enzyme extract 2 mL of 50 mM sodium acetate buffer (pH 4.8), and the mixture was incubated for 60 min at 50°C. It is important to point out that filter paper presents amorphous and crystalline cellulose in a way that filter paper activity can be related to the total cellulolytic activity of the extract. Exo-cellulases activities were

determined using 50 mg of sigmacell cellulose type 20, 20 µm (microcrystalline cellulose, Sigma Aldrich), 1 mL of diluted enzyme extract and 2 mL of 50 mM sodium acetate buffer (pH 4.8). The mixture was incubated for 5 min at 40 °C. Endo-cellulases activities were measured using 1 mL of diluted enzyme extract in 2 mL of a 2% carboxymethyl cellulose (Sigma Aldrich) in a 50 mM acetate buffer (pH 4.8), and the reaction were carried out at 50°C during 30 min. Xylanases activities were measured by adding 0.3 mL of diluted enzyme, 2.7 mL of a 1% beach wood xylan (Sigma Aldrich) solution in a 10 mM phosphate buffer (pH 5.2), and it was incubated for 5 min at 50°C. For all enzyme activity measurements, a standard without substrate was carried out to subtract the initial amount of reducing sugars (RS). Reducing sugars were measured by the spectrophotometric DNS method, using glucose as standard for FPU, exo-cellulases and endo-cellulases whereas xylose was used as standard for xylanase activity. In all cases the absorbance of samples were measured at 540 nm (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme which forms 1 µmol of glucose or xylose per min under assay conditions.

Statistical analysis

All the results were analyzed using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 90%.

Results and discussion

Production of cellulolytic enzymes

Table 2 presents the results obtained in the three PB designs to investigate the production of cellulolytic enzymes using sewage sludge, rice straw and sugarcane bagasse as substrates. The highest values for filter paper activities were using sugarcane bagasse as substrate, whereas sewage sludge was not a good support media for production of extracts with high filter paper activities. However, sewage sludge was an excellent media for production of extracts with high exo-cellulase and xylanase activities. The production of cellulolytic enzymes with endo-cellulase activities was similar in almost whole substrates tested. For run 11 in rice straw substrate and run 14 in sewage sludge substrate were observed endo-cellulase activities of 40.75 $U.g^{-1}$ and 35.32 $U.g^{-1}$, respectively.

Data of Table 2 were used to compute the main effects of independent variables on the production of each cellulolytic enzyme for three substrates used and the results are presented in Table 3. Using rice straw as substrate the moisture content influenced significantly the production of cellulolytic enzymes with filter paper and xylanase activities, both negatively. The production of enzymes with exo-cellulase activities was influenced negatively by CSL and inoculum density. By other hand, inoculums density presented positive effect on production of enzymes with xylanase activities. Using sewage sludge as substrate none of the variables investigated was statistically significant in the evaluated range. Using sugarcane bagasse, CSL presented negative effect on production of enzymes with filter paper activity, whereas pH showed positive effects on production of xylanase.

From the analysis of effects and data of Table 2, it is evident that depending of the experimental condition used, the enzyme produced can present a specific activity. In recent years, there is a growing interest for cellulases to hydrolyze cellulose for bioethanol production. In this scenario, the cellulolytic enzymes should present high filter paper activity since it represents the action of enzyme on amorphous and crystalline cellulose. For this reason, condition of runs 9, 7 and 12 were considered to be optimal for cellulolytic enzymes production using rice straw, sewage sludge and sugarcane bagasse, respectively because in these runs the filter paper activities were the highest for each substrate. The filter paper exo-cellulase, xylanase and endo-cellulase activities using rice straw as substrate were 1.06 $FPU.g^{-1}$, 4.01 $U.g^{-1}$, 71.67 $U.g^{-1}$ and 0.83 $U.g^{-1}$; for sewage sludge the activities obtained were 0.28 $FPU.g^{-1}$, 126.14 $U.g^{-1}$, 51.85 $U.g^{-1}$ and 1.24 $U.g^{-1}$; for sugarcane bagasse the activities were 3.82 $FPU.g^{-1}$, 19.98 $U.g^{-1}$, 9.15 $U.g^{-1}$ and 4.52 $U.g^{-1}$,respectively.

To validate these results, three additional fermentations were carried out at optimal conditions for each substrate. The filter paper exo-cellulase, xylanase and endo-cellulase activities using rice straw as substrate were 0.11 $FPU.g^{-1}$, 13.75 $U.g^{-1}$, 1184.20 $U.g^{-1}$ and 1.07 $U.g^{-1}$; for sewage sludge the activities obtained were 0.17 $FPU.g^{-1}$, 9.96 $U.g^{-1}$, 1168.92 $U.g^{-1}$ and 4.43 $U.g^{-1}$; for sugarcane bagasse the activities were 3.43 $FPU.g^{-1}$, 12.22 $U.g^{-1}$, 8.77 $U.g^{-1}$ and 2.29 $U.g^{-1}$respectively. For sugarcane bagasse there is a good agreement with the results presented in Table 2 at run 12, whereas for sludge sewage and rice straw some deviation was verified. The difference verified for cellulolytic enzymes produced using sewage sludge and rice straw can be the difficulties to maintain the uniformity of media, mainly the moisture content. In practice, the microorganism is a good xylanase producer and this result is corroborated with other studies available in the literature (Ferraz et al. 2003; Heidorne et al. 2006; Chmelová et al. 2011; Chmelová and Ondrejovič 2012).

In a study conducted to evaluate the enzymatic activities of *C. subvermispora* grown on wood chips under solid-state fermentation xylanase was the main hydrolytic activity, reaching its maximum on the 15th day of biodegradation (960 IU per culture). Although reducing sugars have been detected by incubation of filter paper with the culture extracts, cellulase levels were very low and did not attain appropriated concentrations to permit determinations of filter paper activities based on the standard assay. β-glucosidase activities were also low (21 IU per culture on the 15th day) as compared to the xylanase activity (Ferraz et al. 2003).

Only small amounts of reducing sugars were detected by incubation of filter paper strips with the extracts culture of *Ceriporiopsis subvermispora* during biopulping of *Pinus taeda* wood chips. Xylanolytic activity of the extract was initially assayed by measuring the amounts of reducing end groups released after birch xylan treatment with the extract. The level of xylanases was 303 ± 33 IU/L of extract, corresponding to 778 ± 92 IU/kg of wood present in the cultures (Souza-Cruz et al. 2004). Furthermore, three different substrates (wheat straw, pine and poplar wood) were used for producing ligocellulolytic enzymes by *Ceriporiopsis subvermispora*, the authors found that cellulases activities were very low while xylanases predominated. Wheat straw was best substrate for production of cellulases using submerged fermentation (4.38 U/mL) and xylanases (23.34 U/mL) (Chmelová and Ondrejovič 2012).

Several hydrolytic enzymes were produced by *C. subvermispora* during solid state fermentation of *Eucalyptus grandis* and *Pinus taeda* wood chips. Xylanase activities recovered from biotreated *P. taeda* (256.7 U/kg), were almost twice the activities recovered from *E. grandis* (126.2 U/kg). Small amounts of β-xylosidase (2.6 and 4.4 U/kg) and endoglucanase (31.2 and 15.3 U/kg) activities were detected in the *E. grandis* and *P. taeda*

cultures, respectively. The amounts of β-glucosidase produced in *P. taeda* and *E. grandis* were almost the same (46±8 UI/kg) (Heidorne et al. 2006).

In another study cellulase activity was less than 20 IU/kg wood decayed by *C. subvermispora.* Xylanase activity was detected only after 15 days of *E. globulus* biodegradation, 50 IU/kg wood with 15 days and up to 550 IU/ kg wood after 60 days of biotreatment (Mendonça et al. 2008). Delignification of corn stover by the white rot fungus *Ceriporiopsis subvermispora* in solid-state cultivation was evaluated for improving subsequent enzymatic hydrolysis by Wan and Li (2010). In this study for major hydrolytic enzymes, xylanase was the only enzyme detected. Furthermore, reducing sugars released from the assay mixture by incubation of filter paper strips with enzyme extract were not high enough to determine filter paper activity. This result is consistent with several reports on cellulolytic activity of *C. subvermispora* cultured on woody biomass. The pattern of xylanase reached its peak value of 4.8 IU/g solid on day 7. The lowest level was observed on day 18, after which a slight increase in activity followed and the activity reached 4.1 IU/g solid at the end of the fungal pretreatment. The level of xylanase activity in this study was close to that observed from some other solid cultures of *C. subvermispora.* Xylanase activity of 4.5 and 4.3 IU/g solid was observed from 30 days solid fermentation of sugarcane bagasse and hardwood *P. taeda*, respectively (Costa et al. 2005; Guerra et al. 2003). As regards their industrial applicability, xylanases have been used in the feed of monogastric animals to hydrolyze nonstarch polysaccharides such as arabinoxylans, are therefore used in the industry of animal feed (Kuhad et al. 2011).

On the other hand, the highest values founded for cellulases activity in the three different substrates could be used for many industrial applications. In run 11 was observed a high exo-cellulase (143.45 $U.g^{-1}$) and endo-cellulase (40.75 $U.g^{-1}$) activity using rice straw as substrate, both results have potential for detergent industries. In the patent published by Bjork

et al. (1997) a detergent composition containing *Trichoderma longibrachiatum* exo-cellobiohydrolase I and endoglucanase were added at the ratios of 10:1–400:1 to provide superior cleaning capacity (91% reflectance), imparting high softness to the cloths and reduced tensile strength. The endo-glucanases have a wide spectrum of applications in various industries, the high activity this enzyme verified in runs 5 (227.97 $U.g^{-1}$) and 6 (206.57 $U.g^{-1}$) have a potential for food processing industries. Carboxy metyl cellulase was added to the dought to improve bread quality, addition of 250 IU of enzyme for every 100 g of flour improved the maximal volume (635±25.30 cm^3), specific volume (3.99±0.14cm^3/g) and farinographic parameters (water absorption, arrival time, dough development time and dough stability time) of the bread (Nadeem et al. 2009). Mixtures of endo-glucanases and hemicellulases have also been used for biomodification of fiber properties in the paper mills in the beating of pulp (Dienes et al. 2004), because endo-glucanases have to ability to decreased the pulp viscosity with a lower degree of hydrolysis (Pere et al. 1995).

Conclusions

The results obtained in this work allowed concluding that the highest values for the total cellulolytic activity by the method of filter paper (FP) were obtained using sugarcane bagasse as substrate, reaching the value of 3.82 $FPU.g^{-1}$. The sewage sludge was an excellent medium for the production of xylanase and exo-cellulases, reaching peak activity of 227.97 $U.g^{-1}$ and 134.25 $U.g^{-1}$, respectively. The endo-cellulase activity was similar in almost whole substrates tested, as showed in the runs 11 for rice straw (40.75 $U.g^{-1}$) and 14 for sewage sludge (35.32 $U.g^{-1}$). The fungal *G. subvermispora* isolated first time in Latin American is a good producer

of xylanase regardless of the substrate and has the biotechnology potential to produce cellulolytic enzymes in bioreactors.

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Table 1. Matriz Plackett & Burman (PB) design employed to determine the better conditions for cellulolytic enzymes production by *Gelatoporia subvermispora* using different substrates.

Experimento	pH	Pepton	Moisture	CSL*	Inoculum
1	(+1) 7	(-1) 5	(+1) 80	(-1) 0	(-1) 1
2	(+1) 7	(+1) 20	(-1) 60	(+1) 15	(-1) 1
3	(-1) 5	(+1) 20	(+1) 80	(-1) 0	(+1) 3
4	(+1) 7	(-1) 5	(+1) 80	(+1) 15	(-1) 1
5	(+1) 7	(+1) 20	(-1) 60	(+1) 15	(+1) 3
6	(+1) 7	(+1) 20	(+1) 80	(-1) 0	(+1) 3
7	(-1) 5	(+1) 20	(+1) 80	(+1) 15	(-1) 1
8	(-1) 5	(-1) 5	(+1) 80	(+1) 15	(+1) 3
9	(-1) 5	(-1) 5	(-1) 60	(+1) 15	(+1) 3
10	(+1) 7	(-1) 5	(-1) 60	(-1) 0	(+1) 3
11	(-1) 5	(+1) 20	(-1) 60	(-1) 0	(-1) 1
12	(-1) 5	(-1) 5	(-1) 60	(-1) 0	(-1) 1
13	(0) 6	(0) 12,5	(0) 70	(0) 7,5	(0) 2
14	(0) 6	(0) 12,5	(0) 70	(0) 7,5	(0) 2
15	(0) 6	(0) 12,5	(0) 70	(0) 7,5	(0) 2

*Corn steep liquor.

Table 2. Results obtained in the PB design to evaluate the production of cellulolytic enzymes by solid-state fermentation by *Gelatoporia subvermispora* in different solid agro-lignocellulose wastes.

	Rice straw				Sewage sludge				Sugarcane bagasse			
Exp	**R1**	**R2**	**R3**	**R4**	**R1**	**R2**	**R3**	**R4**	**R1**	**R2**	**R3**	**R4**
1	0.00	25.45	14.06	0.44	0.07	118.45	16.83	0.88	1.14	6.69	143.79	1.33
2	0.21	18.53	33.93	0.66	0.07	113.88	20.36	2.90	0.40	107.17	55.15	0.00
3	0.06	24.49	10.60	0.83	0.05	74.81	18.49	3.06	0.33	4.46	21.54	0.89
4	0.02	13.28	1.45	1.06	0.17	70.45	72.32	0.51	0.15	0.00	13.95	1.73
5	0.10	3.12	57.27	1.50	0.13	65.15	227.97	0.46	0.61	21.65	44.54	1.28
6	0.10	24.89	9.93	0.61	0.09	78.03	206.57	0.41	0.34	3.34	77.14	0.16
7	0.10	16.52	1.00	0.89	0.28	126.14	51.85	1.24	0.30	39.52	21.99	0.61
8	0.15	12.50	12.39	1.50	0.00	100.79	35.84	0.20	0.39	3.57	21.43	0.55
9	1.06	4.01	71.67	0.83	0.00	117.73	36.16	0.83	0.09	100.47	27.24	2.56
10	0.49	13.50	104.38	1.33	0.07	82.40	21.92	0.20	1.38	6.69	24.22	4.35
11	0.46	143.45	0.00	40.75	0.06	134.25	22.75	0.77	0.33	40.19	23.44	1.84
12	0.31	75.24	0.89	0.27	0.00	61.61	15.58	0.15	3.82	19.98	9.15	4.52
13	0.08	43.23	10.22	0.72	0.08	97.30	4.11	28.26	0.62	8.57	28.24	0.74
14	0.09	34.47	13.50	0.61	0.07	93.30	4.36	35.32	0.63	8.93	25.56	0.63
15	0.10	36.07	11.94	0.68	0.06	99.49	5.05	30.05	0.61	8.93	22.55	0.69

R1 = Filter paper activity ($U.g^{-1}$); R2 = Exo-cellulases activity ($U.g^{-1}$); R3 = Xylanases activity ($U.g^{-1}$); R4 = Endo-cellulases activity ($U.g^{-1}$)

Table 3. Effects of independent variables on the activities of cellulolytic enzymes produced by solid-state fermentation using rice straw, sewage sludge and sugarcane bagasse as substrates.

	Filter paper activity		**Exo-cellulase activity**		**Xylanase activity**		**Endo-cellulase activity**	
	Effect	**p-value**	**Effect**	**p-value**	**Effect**	**p-value**	**Effect**	**p-value**
	Rice straw							
pH	-0.20	0.1325	-29.57	0.0818	20.75	0.1038	-6.58	0.2734
Peptone	-0.17	0.2082	14.50	0.3618	-15.35	0.2133	6.63	0.2695
Moisture	**-0.37**	**0.0154**	-23.45	0.1547	**-36.45**	**0.0112**	-6.67	0.2673
CSL	0.04	0.7723	**-39.84**	**0.0269**	6.31	0.5955	-6.30	0.2930
Inoculum	0.14	0.2736	**-34.99**	**0.0456**	**35.82**	**0.0122**	-6.24	0.2968
	Sewage sludge							
pH	0.03	0.3381	-14.49	0.5224	64.22	0.1010	-0.15	0.9839
Peptone	0.06	0.1084	6.81	0.7618	58.22	0.1320	1.01	0.8904
Moisture	0.06	0.1464	-1.06	0.9623	9.53	0.7925	0.17	0.9821
CSL	0.05	0.1695	7.43	0.7408	23.73	0.5167	0.11	0.9879
Inoculum	-0.05	0.1695	-17.65	0.4387	57.88	0.1341	-0.21	0.9766
	Sugarcane bagasse							
pH	-0.21	0.6612	-10.44	0.6705	**39.00**	**0.0645**	-0.35	0.7328
Peptone	-0.78	0.1228	13.16	0.5931	0.67	0.9719	-1.71	0.1225
Moisture	-0.66	0.1798	-39.76	0.1284	19.35	0.3234	-1.55	0.1576
CSL	**-0.90**	**0.0799**	31.84	0.2129	-19.16	0.3279	-1.06	0.3183
Inoculum	-0.50	0.3014	-12.23	0.6190	-8.56	0.6550	-0.04	0.9691

Bold variables are statistically significant at 90% of confidence ($p<0.1$)

ULTRASOUND-ASSISTED ENZYMATIC HYDROLYSIS OF NON-TREATED SUGARCANE BAGASSE USING *Gelatoporia subvermispora* CELLULASES OBTAINED BY SOLID-STATE FERMENTATION

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Abstract

Ultrasound-assisted enzymatic hydrolysis of sugarcane bagasse using cellulolytic enzymes produced by the fungal *Gelatoporia subvermispora* was evaluated. Cellulolytic enzymes were produced by solid-state fermentation using three different substrates (rice straw, sewage sludge and sugarcane bagasse) and the crude enzymatic extract obtained in each substrate were employed for hydrolysis experiments, which were carried out using a high-intensity ultrasound probe system of 400 W and 24 kHz. The variables investigated were oscillation amplitude, pulse factor, enzymatic concentration and the influence of substrate for enzyme production on the yield of hydrolysis. The results demonstrated that the substrate used for enzyme production alter the catalytic power of the enzymes, where the best results in terms of yield of hydrolysis were obtained using sewage sludge as substrate for enzyme production. The mean yields obtained with enzymes produced using sewage sludge, sugarcane bagasse and rice straw as substrates were 72.8, 58.7 and 51.2 $g.kg^{-1}$, respectively. Regarding the use of ultrasound to carry out enzymatic reactions, the oscillation amplitude presented a negative effect on yield, whereas pulse factor showed to be benefic for the reactions.

Keywords: Cellulolytic enzymes. Solid-state fermentation. Lignocellulosic material. Yield of hydrolysis.

1 Introduction

As a consequence of the industrial development and population growth, there is an increase of energy consumption in the world. However, conventional energy resources, like fossil fuels, cannot meet the increasing energy demand. The quantities of fossil fuels sources are limited and they contribute to greenhouse gas emissions. In the last years there have been a growing number of studies concerning the use of plant biomass, especially agro-industrial residues, as alternative renewable energy resource and environmentally friendly. Potentially, lignocellulose plant biomass is abundantly available and its supply is renewable and ethanol produced from this kind material can potentially reduce the impacts caused for conventional fuels (Rehman et al., 2013).

Current lignocellulosic ethanol research is driven by the need to reduce the costs of production. The use of the same lignocellulosic biomass for cellulases enzyme production and hydrolysis, improvement in feedstock pretreatment, shortening of fermentation time, could be the basis of reducing production costs (Delabona et al., 2012; Leaes et al., 2013). In recent years, ultrasound power has emerged as an alternative to promote the pretreatment of lignocellulosic materials (Szabó and Csiszár, 2013; Werle et al., 2013). The ability of ultrasound to increase the activity of enzymes and to reduce the mass transfer resistances in the process makes this treatment a potential option for the conversion of lignocellulosic biomass using cellulase for the production of ethanol (Subhedar and Gogate, 2014).

Several authors have shown that saccharification of cellulose is enhanced efficiently by ultrasonic pretreatment (Alvira et al., 2010; Karki et al., 2011; Tian et al., 2012; Pejin et al., 2012). The physiochemical effects of sonication alter the morphology of lignocellulosic biomass particles. Ultrasound is based on the phenomenon of cavitation, which is characterized by the formation, growth and collapse of vapour or gas bubbles (Santos Jr et al.,

2008). Furthermore, mechanical impact produced by the collapse of cavitation bubbles, provide an important benefit of opening up surface area of solid substrates to the action of enzymes (Alvira et al., 2011; Gogate and Kabadi, 2009; Szabó and Csiszár, 2013). However, the effect of ultrasound in the enzymatic hydrolysis of lignocellulosic residues using cellulases is still little studied.

In this sense, the aim of this work was to evaluate the enzymatic hydrolysis of non-treated sugarcane bagasse under indirect sonication using cellulases produced by fungal *Gelatoporia subvermispora*.

2 Materials and Methods

2.1 Microorganism and solid substrates

2.1.1 Microorganism

Gelatoporia subvermispora (Pilát) Niemelä (1985) was first time isolated from Brazilian Pampa Biome in a pine forest by Baldoni, (2012). Stock cultures of *G. subvermispora* were propagated on potato dextrose agar (PDA) using Petri plates at 28°C for five days. . For this purpose, cellulolytic enzymes were produced by solid-state fermentation using three different substrates (rice straw, sewage sludge and sugarcane bagasse) and the crude enzymatic extract obtained in each substrate used for experiments, which were carried out using a high-intensity ultrasound probe system of 400 W and 24 kHz. The variables investigated were oscillation amplitude, pulse factor, enzyme concentration and the influence of substrate for enzyme production on the yield of hydrolysis.

2.1.2 Solid substrate

Rice straw, sewage sludge and sugarcane bagasse were used as substrates for cellulases production and the obtained enzymatic extract was used for ultrasound assisted hydrolysis of sugarcane bagasse.

2.2 Production of cellulolytic enzymes and their assays

2.2.1 Cellulolytic enzymes production

After growing in a pre-culture medium, the microorganism was inoculated in 70 g of substrate in a fixed-bed bioreactor with forced aeration. Solid-state fermentation was carried out using the conditions optimized in a prev with moisture of 40% and 5wt% of pepton to rice straw, with pH 5.0, 5 wt% pepton and moisture of 60% to sugarcane bagasse and pH 5.0, 20 wt% pepton, moisture of 80% and 15% of corn steep liquor to sewage sludge, all experiments at 28°C, during 4 days. After the end of fermentation, the cellulolytic enzymes were extracted using 1500 mL of 50 mM sodium acetate buffer (pH 4.8) in an orbital shaker at 120 rpm and 28°C during one hour and the obtained enzymatic extract for each substrate was stored in a refrigerator at 4°C for posterior use.

2.2.2 Cellulolytic enzymes assays

Cellulolytic enzymes activities were determined as described by Ghose (1987), with few modifications. Exo-cellulase activity was determined using 50 mg of sigmacell cellulose 20 µm - microcrystalline cellulose (Sigma Aldrich), 1mL of diluted enzyme extract, 2 mL of 50 mM sodium acetate buffer (pH 4.8), and the mixture was incubated for 5 min at 40°C. Endo-cellulase activity was measured using 1 mL of diluted enzyme extract in 2 mL of a 2% carboxymethyl cellulose in a 50 mM sodium acetate buffer (pH 4.8), and the reaction were carried out at 50°C during 30 min. The Filter paper activity assay was carried out using 50 mg of filter paper Watmann n°1, 1 mL of diluted enzyme extract 2 mL of 50 mM sodium acetate buffer (pH 4.8), and the mixture was incubated for 60 min at 50°C. Xylanase activity was measured by adding 0.3 mL of diluted enzyme, 2.7 mL of a 1% Beachwood xylan solution in a 10 mM phosphate buffer (pH 5.2), and it was incubated for 5 min at 50°C. For each enzyme activity measurement, a standard without substrate was carried out to subtract the initial amount of reducing sugars (RS). Reducing sugars were measured by the spectrophotometric DNS method, using glucose as standard for filter paper, exo-cellulase and endo-cellulase activities, whereas xylose for xylanase activity. The absorbance was measured at 540 nm (Miller, 1959). One unit of enzyme activity is the amount of enzyme which forms 1 µmol of glucose or xylose per min at respective temperature of reaction during the hydrolysis reaction.

2.3 Enzymatic hydrolysis of non-treated sugarcane bagasse

The experimental system for indirect sonication evaluation was composed of a sonication beaker with a capacity for 5 test tubes of 15 mL, acting as micro-reactors, a

thermostatic water bath (temperature accuracy of ±1.0°C) and a high-intensity ultrasound probe system of 400 W and 24 kHz with amplitude adjustable from 20 to 100% and pulse adjustable from 0 to 100% (Model UP 400S, Hielscher, Germany), equipped with a 22 mm microtip up.

The sonication beaker with test tubes was immersed in the thermostatic bath during hydrolysis to maintain constant the temperature and the sonication probe was coupled in the middle of the beaker in direct contact with water while the hydrolysis were carried out inside the test tubes, characterizing the process with indirect sonication.

All hydrolysis experiments were carried out at 50°C±2°C, temperature previously considered the best for sugarcane bagasse hydrolysis using the produced enzymatic extract. After adding the substrate and enzyme extract, the final volume was adjusted with 50 mM sodium acetate buffer solution in order to maintain the medium pH at 4.8. The final volume for direct sonification experiments was 10 mL. The solid liquid ratio for all experiments was 0.05 wt% and 1% of Tween 80 was added to enhance cellulose hydrolysis yields by reducing non-productive enzyme adsorption on the substrate.

After the hydrolysis, an aliquot of solution was filtered by vacuum filtration (Whatman qualitative filter paper, grade 1) and the supernatant was used to determine the amount of fermentable sugars by the 3.5-dinitrosalicylic acid method (DNS). The results were expressed in terms of gram of fermentable sugars per kilogram of dry solid material, which represent the yield of hydrolysis. All the results were analyzed using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 90%. The effects of concentration of crude enzymatic extract (5.0 – 25.0 v/w), oscillation amplitude (20 – 40%) and pulse cycle (0.4 to 1.0) on the hydrolysis of sugarcane bagasse were assessed by means of central composite rotational design (CCRD) with 15 runs plus 3 central points for each cellulolytic enzymes.

3 Results and Discussion

Table 1 presents the yield of hydrolysis of non-treated sugarcane bagasse after indirect exposure to ultrasound for each cellulolytic enzymatic complex tested in this work. If considered the mean yield for each cellulolytic enzymatic pool tested, the highest yields were obtained using the enzymes produced using sewage sludge, since the mean yield (considering 17 runs of the CCRD) was 72.8 $g.kg^{-1}$, whereas for enzyme obtained using sugarcane bagasse and rice straw the mean yields were 58.7 and 51.2 $g.kg^{-1}$, respectively. The variation in the mean yield can be associated with the differences in the activities of the enzymes present in each crude extract. Sewage sludge was an excellent media for production of extracts with high exo-cellulase and xylanase activities. On the other hand, for sugarcane bagasse was observed lower values for the activity of filter paper. The production of cellulolytic enzymes with endo-cellulase activities was similar in almost whole substrates tested. For run 11 in rice straw substrate and run 14 in sewage sludge substrate were observed endo-cellulase activities 40.75 $U.g^{-1}$ and 35.32 $U.g^{-1}$, respectively.

Data of Table 1 were used to determine the effects of the studied variables on yield of fermentable sugar for each enzymatic extract. To organize the presentation and discussion, the effects will be presented individually for each extract. Fig. 1 presents the effects, in the form of Pareto chart, for hydrolysis carried out using the enzymatic pool produced with sugarcane bagasse as substrate. Linear effects for enzyme concentration and pulse factor were statistically significant, all of them positives, with 90% of significance ($p<0.1$), while the linear effect for oscillation amplitude and the interaction effect between oscillation amplitude and pulse factor was statistically significant, but showed a negative effect on hydrolysis yield. The highest hydrolysis yield (103.6 $g.kg^{-1}$) was obtained in experiment 14, for enzyme concentration of 75 $\mu L.g^{-1}$, oscillation amplitude of 30% and high pulse factor (1.0), followed

by the experiment 6 (99.3 $g.kg^{-1}$) at level +1 of the CCRD for enzyme concentration and pulse factor and level -1 for oscillation amplitude. It is important to highlight that the best results in terms of yield were obtained in the runs where enzyme concentration and pulse factor were maintained at level +1 or superior, while oscillation amplitude at low level, corroborating with analysis of the effects showed in Figure 1.

Condition 3 (144.5 $g.kg^{-1}$) for sewage sludge, 17 (104.2 $g.kg^{-1}$) for rice straw and 14 (103.6 $g.kg^{-1}$) for sugarcane bagasse had the highest yields of hydrolysis assisted by indirect sonication and were compared to results obtained using only the water bath without ultrasound. The hydrolysis yield obtained only with bath using sewage sludge, rice straw and sugarcane bagasse were 152.5 $g.kg^{-1}$, 138 $g.kg^{-1}$ and 143 $g.kg^{-1}$, respectively. Results clearly prove that even sonication has a significant effect on the enzyme activity, the lowest yield hydrolysis obtained with ultrasound occur because selected conditions have the pulse factor at level 0 or superior. This statement is in accordance with the results published in a previous study (Szabó and Csiszár, 2013), where the filter paper activity of the enzyme sonicated with 40% amplitude decreased to 73 FPU/mL, wich represents a 12% reduction. By increasing the amplitude to 60% and 80%, the enzyme activity decreased to 67 FPU/mL (80% of the original) and 63 FPU/mL (75% of the original), respectively.

Sensitivity of the enzyme macromolecules to sonication is different. The alteration in enzyme activity that occurred during sonication depended largely on both the characteristics of the enzyme and the sonication system (Macleod and Dunn, 1967). We can conclude from the results that sonication the enzyme solution at different amplitudes, the cellulase enzyme underwent changes, and the reduction of the enzyme activity which occurred was highly dependent on the amplitude. Consequently, optimization of the parameters of the sonicated system is required specifically for each of the enzymes used.

The influence of independent variables on the yield of hydrolysis using the cellulolytic enzymes obtained from solid-state fermentation of sugarcane bagasse can be better visualized in Figure 2, which was obtained from empirical model presented in Eq. 1:

$$FS_{SB} = 80.36 + 19.57 \cdot E - 18.88 \cdot A - 18.77 \cdot A^2 + 35.54 \cdot P - 28.63 \cdot P \cdot A \qquad (1)$$

where FS_{SB} is the yield of fermentable sugar ($g.kg^{-1}$) obtained for the indirect sonication hydrolysis using cellulolytic enzymes from sugarcane bagasse, P, A and E are the coded pulse factor, oscillation amplitude and enzyme concentration, respectively. This model was validated by analysis of variance (ANOVA) and the determination coefficient (r^2) was 0.8427. Analyzing contour plots referring to influence of enzyme concentration and oscillation amplitude (Figure 2a) it is possible to note that high yield can be achieved in the region of high enzyme concentration and low values of oscillation amplitude. Regarding the effects of enzyme concentration and pulse factor (Figure 2b), the best results were also obtained for high enzyme concentration combined with high values of pulse factor. In contour plots for oscillation amplitude and pulse factor (Figure 2c) high yields were obtained at higher values of pulse factor and lower values of oscillation amplitude.

Figure 3 presents the effects of the independent variables on the yield of hydrolysis using cellulolytic enzymes obtained from solid-state fermentation of rice straw. The quadratic effects for enzyme concentration, oscillation amplitude and pulse factor were statistically significant ($p<0.1$), all of them negatives. The negative effects of quadratic terms are related to a presence of maximum point for each variable within the studied range. To identify these maximum points an empirical model was generated considering the significant terms plus the interaction of oscillation amplitude and pulse factor, which is presented in Eq. 2:

$$FS_{RS} = 98.34 + -48.58 \cdot E^2 - 46.38 \cdot A^2 - 22.57 \cdot P^2 + 27.05 \cdot P \cdot E \qquad (2)$$

where FS_{RS} is the yield of fermentable sugar ($g.kg^{-1}$) obtained for the indirect sonication hydrolysis using cellulolytic enzymes from rice straw, P, A and E are the coded pulse factor,

oscillation amplitude and enzyme concentration, respectively. This model was validated by analysis of variance (ANOVA) and the determination coefficient (r^2) was 0.8201. The applicability of Eq. 2 is presented in Figure 4, where it is seen that maximum yield of hydrolysis can be obtained around of central point of each variable.

Figure 5 presents the effects of the independent variables on the yield of hydrolysis using cellulolytic enzymes obtained from solid-state fermentation of sewage sludge. Only linear effects of available variables were statistically significant. The interaction between oscillation amplitude and pulse factor was statistically significant, but showed a negative effect on hydrolysis yield. In the same way that for enzymes obtained for sugarcane bagasse and rice straw, was generated an empirical model from data of Table 1, but this model was not validated by analysis of variance (ANOVA) and, for this reason, it was not presented here.

4 Conclusions

In this work were used cellulolytic enzymes from fungal *Gelatoporia subvermispora* produced by solid-state fermentation using different substrates to hydrolyze non-treated sugarcane bagasse using indirect sonication. The results demonstrated that the substrate used for enzyme production alter the catalytic power of the enzymes, where the best results were obtained using sewage sludge as substrate for enzyme production. The mean yields obtained with enzymes produced using sewage sludge, sugarcane bagasse and rice straw as substrates were 72.8, 58.7 and 51.2 $g.kg^{-1}$, respectively. Regarding the use of ultrasound to carry out enzymatic reactions, the oscillation amplitude presented a negative effect on yield, whereas pulse factor showed to be benefic for the reactions.

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LIST OF FIGURE CAPTIONS

Figure 1. Pareto chart expressing the effects of process variables on the enzymatic hydrolysis optimization under indirect sonication using sugarcane bagasse as substrate for cellulolytic enzyme production. P – pulse; E – enzyme concentration; A – oscillation amplitude.

Figure 2. Contour plots for enzymatic hydrolysis optimization of crude sugarcane bagasse under indirect sonication using sugarcane bagasse as substrate for cellulolytic enzyme production: (a) enzyme concentration x oscillation amplitude, (b) enzyme concentration x pulse factor, (c) oscillation amplitude x pulse factor.

Figure 3. Pareto chart expressing the effects of process variables on the enzymatic hydrolysis optimization under indirect sonication using rice straw as substrate for cellulolytic enzyme production. P – pulse; E – enzyme concentration; A – oscillation amplitude.

Figure 4. Contour plots for enzymatic hydrolysis optimization of crude sugarcane bagasse under indirect sonication using rice straw as substrate for cellulolytic enzyme production: (a) enzyme concentration x oscillation amplitude, (b) enzyme concentration x pulse factor, (c) oscillation amplitude x pulse factor.

Figure 5. Pareto chart expressing the effects of process variables on the enzymatic hydrolysis optimization under indirect sonication using sewage sludge as substrate for cellulolytic enzyme production. P – pulse; E – enzyme concentration; A – oscillation amplitude.

Table 1. Independent and dependent variables in CCRD under indirect sonication (IS) using sugarcane bagasse (SB), rice straw (RS) and sewage sludge (SS) as substrate.

Run	E (% v/w)	A (%)	P (%)	Yield - SB ($g.kg^{-1}$)	Yield - RS ($g.kg^{-1}$)	Yield - SS ($g.kg^{-1}$)
1	9.0 (-1)	24 (-1)	0.5 (-1)	16.5	74.0	79.8
2	21.0 (1)	24 (-1)	0.5 (-1)	20.2	36.7	9.5
3	9.0 (-1)	36 (1)	0.5 (-1)	12.8	75.2	144.5
4	21.0 (1)	36 (1)	0.5 (-1)	63.2	47.7	130.5
5	9.0 (-1)	24 (-1)	0.9 (1)	79.2	14.4	89.5
6	21.0 (1)	24 (-1)	0.9 (1)	99.3	27.5	67.2
7	9.0 (-1)	36 (1)	0.9 (1)	25.1	49.8	76.7
8	21.0 (1)	36 (1)	0.9 (1)	38.2	80.1	101.4
9	5.0 (-1.68)	30 (0)	0.7 (0)	53.2	5.8	60.0
10	25.0 (1.68)	30 (0)	0.7 (0)	80.7	22.3	61.0
11	15.0 (0)	20 (-1.68)	0.7 (0)	82.5	11.3	35.7
12	15.0 (0)	40 (1.68)	0.7 (0)	51.0	23.0	77.0
13	15.0 (0)	30 (0)	0.4 (-1.68)	36.1	59.6	32.7
14	15.0 (0)	30 (0)	1.0 (1.68)	103.6	41.9	67.5
15	15.0 (0)	30 (0)	0.7 (0)	76.1	101.4	65.1
16	15.0 (0)	30 (0)	0.7 (0)	87.1	95.0	61.7
17	15.0 (0)	30 (0)	0.7 (0)	73.3	104.2	65.1

E - enzyme concentration; A - oscillation amplitude; P - pulse factor.

SB, RS and SS are referring to enzyme produced using sugarcane bagasse, rice straw and sewage sludge as substrate.

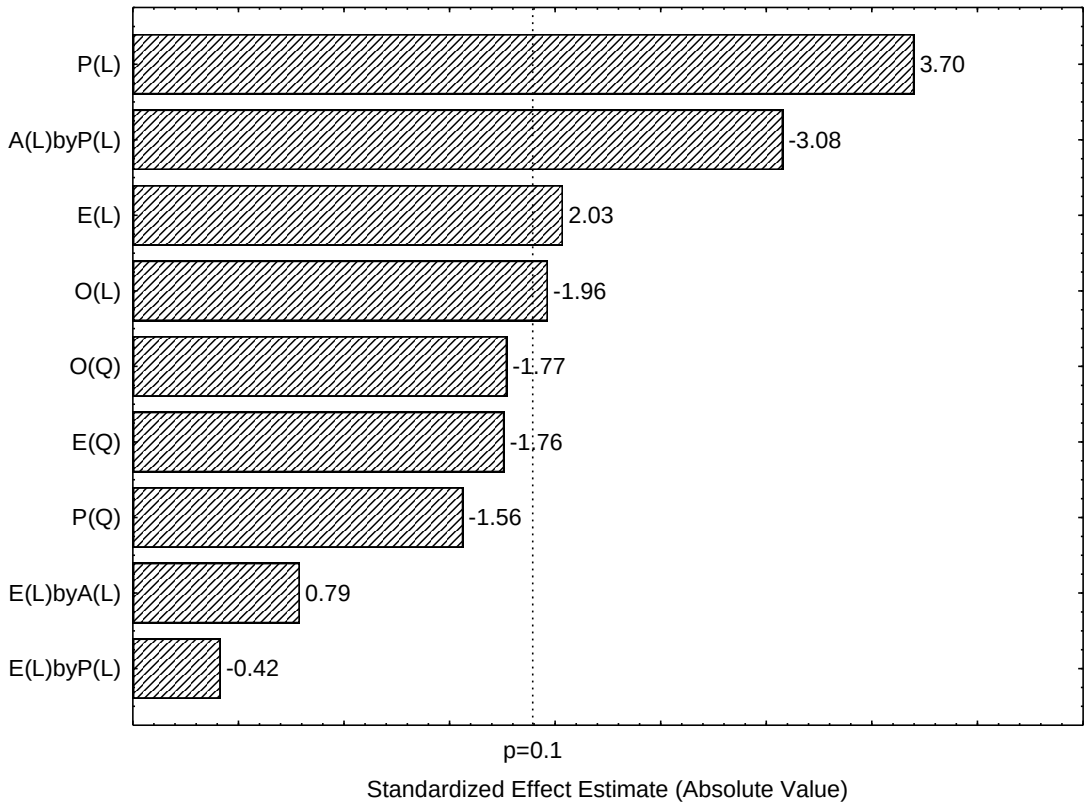
P(L)
A(L)byP(L)
E(L)
O(L)
O(Q)
E(Q)
P(Q)
E(L)byA(L)
E(L)byP(L)
3.70
-3.08
2.03
-1.96
-1.77
-1.76
-1.56
0.79
-0.42
p=0.1
Standardized Effect Estimate (Absolute Value)

Figure 1

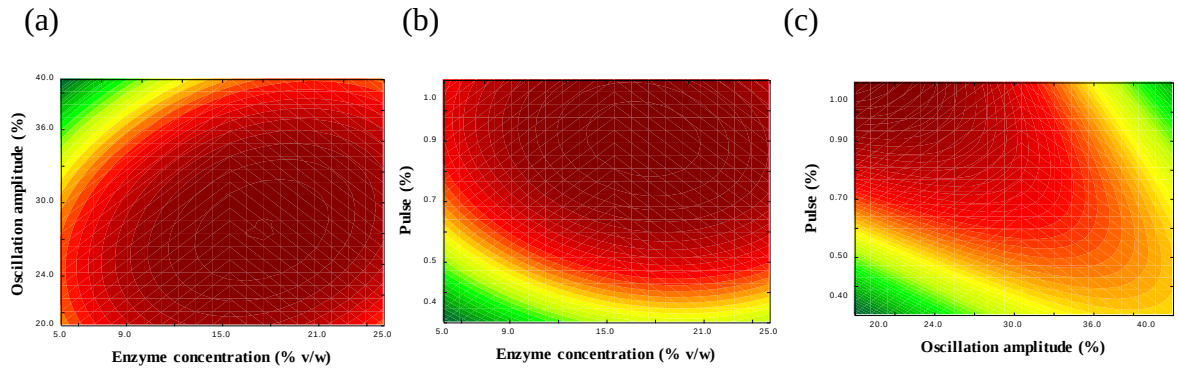
(a)
(b)
(c)
Oscillation amplitude (%)
40.0
36.0
30.0
24.0
20.0
5.0
9.0
15.0
21.0
25.0
Enzyme concentration (% v/w)
Pulse (%)
1.0
0.9
0.7
0.5
0.4
5.0
9.0
15.0
21.0
25.0
Enzyme concentration (% v/w)
Pulse (%)
1.00
0.90
0.70
0.50
0.40
20.0
24.0
30.0
36.0
40.0
Oscillation amplitude (%)

Figure 2

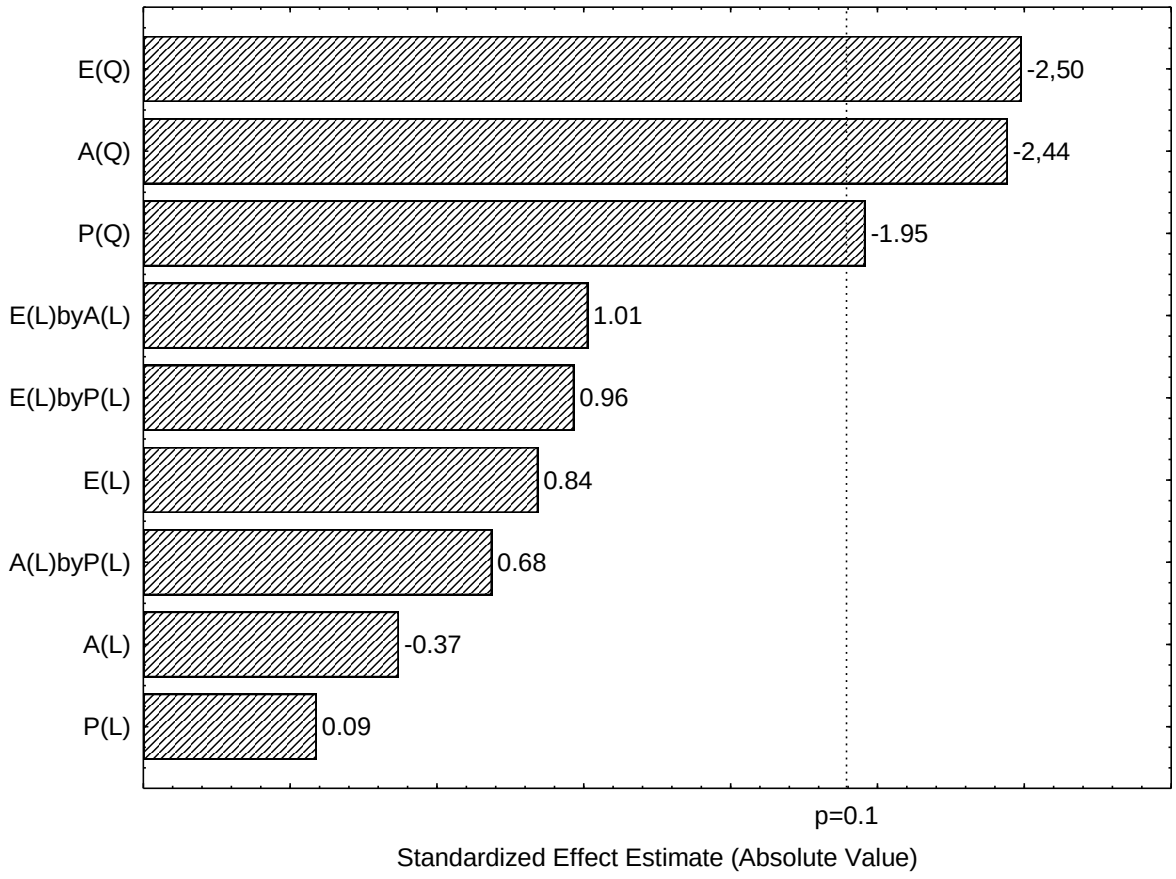
E(Q)
A(Q)
P(Q)
E(L)byA(L)
E(L)byP(L)
E(L)
A(L)byP(L)
A(L)
P(L)
-2,50
-2,44
-1.95
1.01
0.96
0.84
0.68
-0.37
0.09
p=0.1
Standardized Effect Estimate (Absolute Value)

Figure 3

(a) (b) (c)

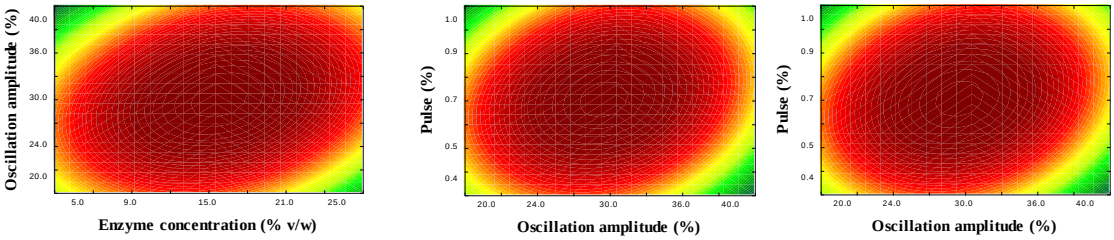
Oscillation amplitude (%)
40.0
36.0
30.0
24.0
20.0
5.0
9.0
15.0
21.0
25.0
Enzyme concentration (% v/w)
Pulse (%)
1.0
0.9
0.7
0.5
0.4
20.0
24.0
30.0
36.0
40.0
Oscillation amplitude (%)
Pulse (%)
1.0
0.9
0.7
0.5
0.4
20.0
24.0
30.0
36.0
40.0
Oscillation amplitude (%)

Figure 4

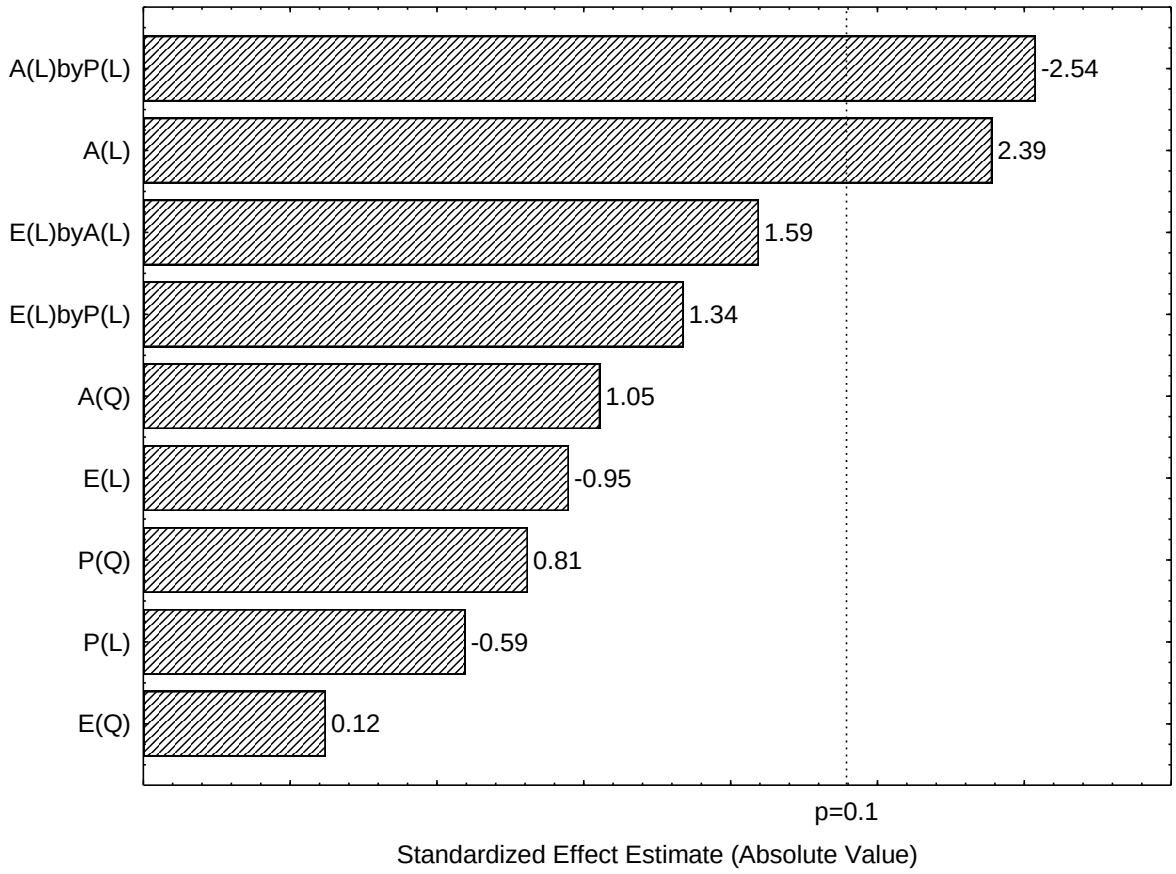
A(L)byP(L)
A(L)
E(L)byA(L)
E(L)byP(L)
A(Q)
E(L)
P(Q)
P(L)
E(Q)
-2.54
2.39
1.59
1.34
1.05
-0.95
0.81
-0.59
0.12
p=0.1
Standardized Effect Estimate (Absolute Value)

Figure 5

DISCUSSÃO

Para determinar a produção de celulases pelo fungo *G. subvermispora*, os resíduos palha de arroz, bagaço de cana e lodo de esgoto foram utilizados como substratos para fermentações em estado sólido. O fungo *G. subvermispora* apresentou alta atividade celulolítica total determinada pelo método de papel filtro (PF) utilizando bagaço de cana como substrato. Para a produção de enzimas com atividade de exo-celulases e xilanases, o substrato mais adequado foi o lodo de esgoto, enquanto a produção de enzimas com atividade de endo-celulases apresentou valores semelhantes nos três substratos testados.

Com relação à influência das diferentes variáveis observadas na produção de enzimas celulolíticas utilizando a palha de arroz como substrato, o conteúdo de umidade influenciou significativamente a atividade de papel filtro e de xilanases, porém negativamente. A produção de enzimas com atividade de exo-celulases foi influenciada negativamente pela concentração de água de maceração de milho e pela densidade de inóculo. Porém, a densidade de inóculo apresentou um efeito positivo na produção de enzimas com atividade xilanolítica. Utilizando bagaço de cana como substrato, a concentração de água de maceração de milho influenciou negativamente a produção de enzimas com atividade de papel filtro, enquanto o pH apresentou um efeito positivo na produção de xilanases. Por outro lado, nenhuma das variáveis analisadas apresentou efeito significativo sobre a atividade das enzimas utilizando o lodo de esgoto como substrato.

As condições 7, 9 e 12 utilizando os substratos lodo de esgoto, palha de arroz e bagaço de cana, respectivamente, foram consideradas as melhores para a atividade de celulases produzidas pelo fungo *G. subvermispora.* A atividade celulolítica total avaliada pelo método do papel filtro (PF), de exo-celulases, de xilanases e de endo-celulases utilizando a palha de arroz como substrato foi de 1.06 $FPU.g^{-1}$, 4.01 $U.g^{-1}$, 71.67 $U.g^{-1}$ e 0.83 $U.g^{-1}$; para o lodo de esgoto as atividades obtidas foram 0.28 $FPU.g^{-1}$, 126.14 $U.g^{-1}$, 51.85 $U.g^{-1}$ e 1.24 $U.g^{-1}$; para o bagaço de cana as atividades foram 3.82 $FPU.g^{-1}$, 19.98 $U.g^{-1}$, 9.15 $U.g^{-1}$ e 4.52 $U.g^{-1}$, respectivamente.

Para validar os resultados obtidos três fermentações adicionais foram realizadas para cada substrato. O substrato bagaço de cana apresentou resultados semelhantes aos obtidos anteriormente, 3.43 $FPU.g^{-1}$, 12.22 $U.g^{-1}$, 8.77 $U.g^{-1}$ e 2.29 $U.g^{-1}$, para atividade de papel filtro, exo-celulases, xilanases e endo-celulases, respectivamente. Os substratos lodo de esgoto e palha de arroz apresentaram desvios quanto aos resultados anteriores, principalmente

devido às dificuldades de estabilização dos meios fermentativos. O fungo *G. subvermispora* foi um bom produtor de xilanases, este resultado está de acordo com outros estudos realizados (FERRAZ et al., 2003; HEIDORNE et al., 2006; CHMELOVÁ et al., 2011; CHMELOVÁ e ONDREJOVIČ, 2012). As xilanases apresentam aplicabilidade em diversas indústrias, principalmente nas indústrias de ração animal, pois podem ser utilizadas na alimentação de monogátricos atuando na hidrólise de arabinoxilanas (KUHAD et al., 2011).

Além disso, os altos valores de atividade de celulases observados em outras condições experimentais podem apresentar diversas aplicações industriais. Na condição 11 foi observada alta atividade de exo-cellulase (143.45 $U.g^{-1}$) e de endo-cellulase (40.75 $U.g^{-1}$) utilizando a palha de arroz como substrato, podendo ser utilizados na produção de detergentes. Na patente publicada por Bjork et al. (1997), um detergente contendo *Trichoderma longibrachiatum* foi formulado com exo-celobiohidrolase I e endoglucanase para proporcionar maior limpeza e maciez às roupas. As atividades de endo-glucanases verificadas nas condições 5 (227.97 $U.g^{-1}$) e 6 (206.57 $U.g^{-1}$) apresentam potencial para a indústria de processamento de alimentos. Carboximetilcelulase foi adicionada para garantir qualidade na fabricação de pães (NADEEM et al., 2009). Misturas de endo-glucanases e hemicelulases também apresentam potencial para serem utilizadas na biomodificação das fibras nas fábricas de papel (DIENES et al., 2004).

As enzimas produzidas pelo fungo *G. subvermispora* nos três substratos testados, foram utilizadas na hidrólise de bagaço de cana através de sonicação indireta. Considerando os valores médios obtidos para os rendimentos de hidrólise, o maior rendimento de hidrólise foi obtido utilizando lodo de esgoto como substrato (72.8 $g.kg^{-1}$), seguido pelo bagaço de cana (58.7 $g.kg^{-1}$) e pela palha de arroz (51.2 $g.kg^{-1}$).

Com relação à influência da sonicação indireta no rendimento de hidrólise com a aplicação de enzimas produzidas utilizando o bagaço de cana como substrato, os maiores rendimentos foram obtidos quando a concentração da enzima e o fator pulso foram mantidos no nível +1 ou em níveis superiores do delineamento central composto rotacional (DCCR), enquanto a amplitude foi mantida em níveis mais baixos.

Os resultados demonstram um efeito significativo da sonicação indireta sobre a atividade das enzimas. Em um estudo realizado por (SZABÓ e CSISZÁR, 2013), a atividade de papel filtro da enzima sob sonicação com 40% de amplitude, diminuiu para 73 FPU/mL, cerca de 12%. Porém, aumentando a amplitude para 60% e 80%, a atividade caiu para 67 FPU/mL (80% do original) e 63 FPU/mL (75% do original), respectivamente.

Utilizando a palha de arroz como substrato para a produção de enzimas, o efeito quadrático da concentração de enzima, fator pulso e amplitude foram estatisticamente

significativos, porém apresentaram efeito negativo sobre o rendimento de hidrólise. Entretanto, utilizando o lodo de esgoto como substrato, apenas os efeitos lineares das variáveis testadas foram significativos. A interação entre o fator pulso e a amplitude foram estatisticamente significativos, mas também apresentaram um efeito negativo sobre os rendimentos obtidos.

CONCLUSÃO

O isolamento no bioma Pampa e o estudo biotecnológico do fungo *G. subvermispora*, relatado pela primeira vez na América Latina, é um exemplo dos muitos estudos que ainda podem ser realizados visando o conhecimento e a utilização sustentável da diversidade de microrganismos presentes no solo. Em estudo anterior, foi realizada a identificação morfológica e molecular deste organismo e nesse estudo foi possível conhecer o potencial biotecnológico dessa espécie. A produção de celulases pelo fungo *G. subvermispora* utilizando diferentes resíduos lignocelulósicos, através de fermentação em estado sólido, é uma alternativa para tornar a produção de etanol mais viável economicamente e mais sustentável. O fungo *G. subvermispora* produziu celulases nas condições avaliadas utilizando palha de arroz, lodo de esgoto e bagaço de cana como fonte de carbono. Além disso, as celulases produzidas pelo fungo foram eficazes na hidrólise de material celulósico utilizando ultrassom como pré-tratamento. Portanto, *G. subvermispora* apresenta potencial para a produção de celulases em biorreatores, as quais podem hidrolisar materiais celulósicos. Desse modo, as enzimas celulolíticas produzidas por *G. subvermispora* podem ser utilizadas em diversos estudos futuros para determinar quais as melhores condições para sua produção em grande escala e que viabilizem sua aplicação industrial, na produção de detergentes biodegradáveis, na indústria têxtil, na formulação de rações animais, na produção de vinhos e cervejas, e também, na produção de etanol.

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