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INSTITUTO DE PESQUISA EM
BIOENERGIA

PROGRAMA INTEGRADO (UNESP, USP E UNICAMP) DE PÓS-GRADUAÇÃO EM BIOENERGIA

DEVELOPMENT OF BIOPROCESS FOR FIBROLYTIC FUNGAL ENZYMES PRODUCTION FROM LIGNOCELLULOSIC RESIDUES AND ITS APPLICATION ON KRAFT PULP BIOBLEACHING AND XYLOOLIGOSACCHARIDES PRODUCTION

Tania Sila Campioni

Tese apresentada ao Instituto de Pesquisa em Bioenergia de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências.

Orientador(a): Pedro de Oliva Neto

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TÍTULO DA TESE: DEVELOPMENT OF BIOPROCESS FOR PRODUCTION OF FIBROLYTIC FUNGAL ENZYMES FROM LIGNOCELLULOSIC MATERIALS AND ITS APPLICATION ON KRAFT PULP BIOPULPING AND XOS PRODUCTION

AUTORA: TANIA SILA CAMPIONI
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, 02 de agosto de 2018

Á toda a minha família pelo apoio incondicional, dedicação, amor e carinho, que me ajudam a superar todos os obstáculos e a ser uma pessoa melhor a cada dia.

Dedico

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Resumo

Desejando ao final do trabalho obter um bioprocesso integrado usando palha de cana-de-açúcar (PC), este trabalho teve início com a utilização desse substrato para produção de enzimas fribrolíticas, xilanases e celulases, em culturas axênicas, incluindo espécies de *Trichoderma* e *Aspergillus*. A triagem para o melhor produtor foi realizada em “shaker” em fermentação submersa. A cultura do fungo *T. reesei* QM9414 alcançou a melhor produção de enzimas, e em tanque agitado, utilizando um biorreator de 3 L, mostrou o mesmo perfil de produção (~90 U/mL, 0.6 FPU/mL para xilanase e celulases, respectivamente). Em relação a este resultado, a produção de enzimas para as misturas binárias e ternárias destes fungos foi menor, sendo que a melhor combinação, *T. reesei* QM 9414+*A. fumigatus* M51, alcançou 60 U/mL e 0.08 FPU/mL respectivamente. Com intuito de otimizar a produção de enzimas utilizando um mix de substratos: palha de cana, como principal componente, e o farelo de trigo e a polpa cítrica, como supostos indutores de atividade enzimática, foi realizado um delineamento de misturas do tipo D-optimal. O resultado da otimização da mistura dos substratos mostrou que o trigo e a polpa cítrica não tiveram um efeito indutivo na produção das enzimas tendo a palha de cana como principal substrato. A enzima xilanase foi caracterizada em seu pH e temperatura ótimos (pH 5, e 50 ºC respectivamente), bem como a estabilidade da enzima nestes parâmetros. Alguns íons e EDTA foram aplicados para determinar a estabilidade da enzima nessas condições, sendo o melhor indutor o Mn^{2+} com 49% de aumento de atividade (10 mM). O extrato contendo xilanases, produzido nas condições previamente otimizadas foi aplicado no biobranqueamento da polpa Kraft. A otimização da biobranqueamento mostrou uma diminuição do índice Kappa, 12.5% (30 U/g e 30 min), bem como houve a liberação de açúcares e compostos cromóforos. Este tratamento na polpa foi responsável por diminuir em 10% a quantidade de dióxido de cloro utilizado no branqueamento químico, uma vez que sua alvura foi a mesma que o controle sem tratamento enzimático. A xilana presente na PC foi extraída com NaOH por meio de tratamento termo-químico. Após este processo a xilana foi hidrolisada, para a produção de xiloligossacarídeos (XOS) por duas diferentes rotas, com enzimas (utilizando o extrato contendo xilanases), e com ácido fosfórico (95 ºC e 120 ºC). Os melhores ensaios que produziram XOS nas duas rotas não apresentaram diferença significativa, 5.34 e 5.94 g/L correspondendo a 16 e 17.45% de rendimento em XOS. A produção de XOS por via enzimática não formou furfural, entretanto, a hidrólise ácida de XOS é uma alternativa mais rápida. XOS e xilose foram produzidos por meio da hidrólise enzimática da xilana, foram assimilados por bactérias probióticas e por uma levedura produtora de xilanase e celulase. Assim, os resultados mostram que a PC pode ser usada em bioprocessos utilizando microrganismos especiais, visando a produção de enzimas, açúcares fermentecíveis, aproveitamento de resíduos e produção de moléculas nobres tais como o XOS, dentro de um conceito moderno de biorrefinaria desde que outros componentes presentes na PC possam ser utilizados em outros bioprocessos, como produção de bioenergia.

Palavras-chave: Palha de cana-de-açúcar. Enzimas fibrolíticas. Biobranqueamento. Produção de XOS. Biorrefinaria.

Abstract

In order to obtain an integrated bioprocess using Sugarcane Straw (SS), this work began with the use of this substrate for the fibrolytic enzymes production, xylanases and cellulases, in axenic fungal cultures, including *Trichoderma* and *Aspergillus* species. The screening for the best producer was performed in shaker under submerged fermentation. The *T. reesei* QM9414 culture achieved the best enzyme production, and in a stirred tank using a 3 L bioreactor showed the same production profile (~90 U/mL and 0.6 FPU/mL for xylanase and cellulase, respectively). Regarding this result, the enzyme production by binary and ternary mixtures of these fungi was lower, as example the best combination *T. reesei* QM 9414+*A. fumigatus* M51, reached 60 U/mL and 0.08 FPU/mL, respectively. Aiming optimize the enzyme production by a mix of substrates using SS as the main substrate, and wheat bran and citrus pulp as supposed enzyme inductors, a D-optimal mixture design was performed. The mixture substrates optimization showed that wheat bran and citrus pulp did not have an inductive effect on the enzymes production. The enzyme xylanase was characterized by its optimal pH and temperature (pH 5 and 50 ºC, respectively, as well as the stability of the enzyme in these parameters. Some ions and EDTA were applied to determine the xylanase stability under these conditions, and the ion Mn^{2+} was the best inductor, 49% (10 mM). The extract containing xylanases, produced under previous optimized conditions was applied in the Kraft pulp biobleaching. The biobleaching optimization showed a decrease in the Kappa number, 12.5% (30 U/g e 30 min), as well as well as the release of sugars and the presence of chromophores compounds were also observed. This treatment performed in the pulp was responsible the decrease in 10% the chlorine dioxide amount used in the chemical bleaching, since its brightness was the same found in the sample that have no enzymatic treatment. The xylan present in the SS was extracted with NaOH by thermo-chemical treatment. After this, the xylan was hydrolyzed, for the production of xylooligosaccharides (XOS), by two different routes, enzymatic (using the crude extract produced) and acid (95 ºC and 120 ºC). The best tests that produced XOS in both routes did not present significant difference, 5.34 and 5.94 g/L corresponding to 16 e 17.45% of XOS yield. The enzymatic XOS production did not produce furfural, but the acid route is a faster alternative. As products of xylan enzymatic hydrolysis, XOS and xylose, were assimilated by probiotic bacteria and a fibrolytic yeast. Thus, the results showed that SS can be used in bioprocesses using special microorganisms, aiming the production of enzymes, fermentable sugars, waste utilization and noble molecules production,

such as XOS in a modern biorefinery concept since other components of the PC can be used in other bioprocesses, such as bioenergy production.

Keywords: Sugarcane straw. Fibrolytic enzymes. Biobleaching. XOS production. Biorefinery.

INDEX

GENERAL INTRODUCTION

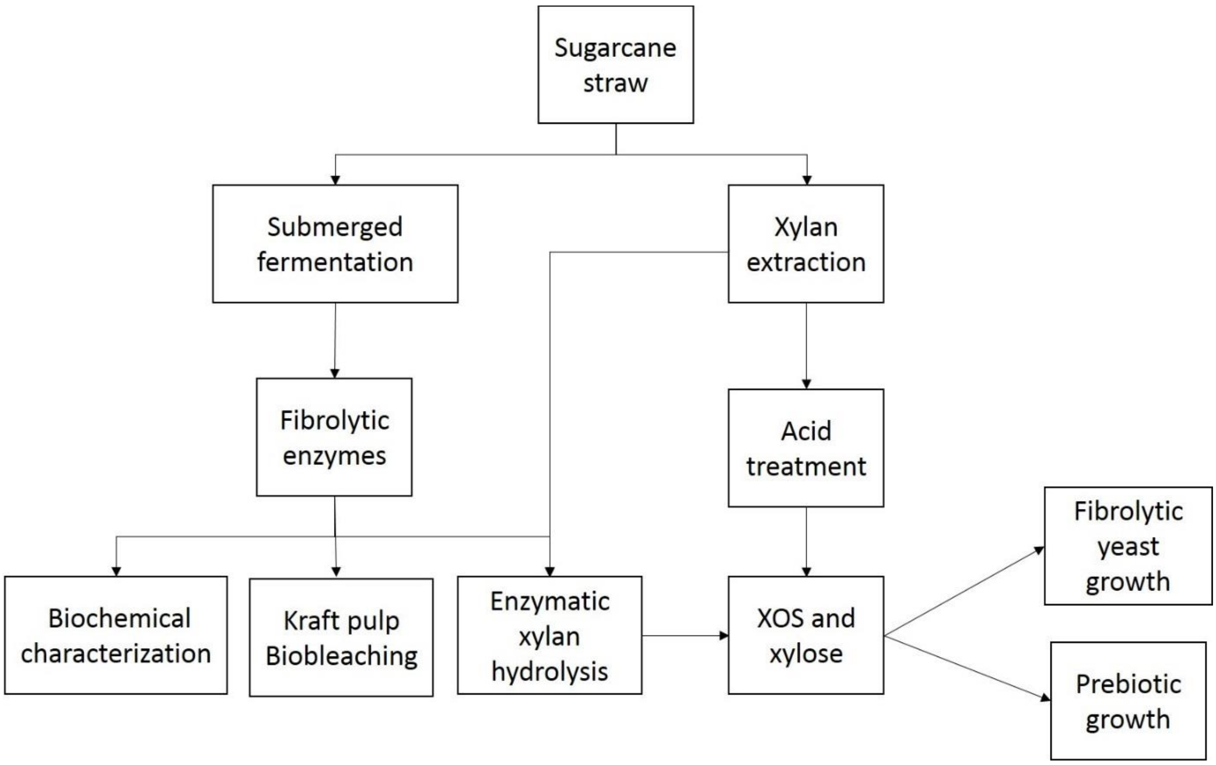
Biotechnology processes are an important route to produce many types of biomolecules in different areas. Investments in research and development of new bioprocesses, mainly focused on industrial enzyme production, are recently highlighted due to the great diversity of their application. Enzymes are being used in a wide range of sectors, especially in the food, paper, biofuel, textile, animal and pharmaceutical industries, with a promising and growing market. In addition, enzymes have other advantages such as the high reaction specificity, which contributes to the process efficiency, are biological products and can have its activity regulated, and still act in low concentrations under mild conditions of pH and temperature. On the other hand, the enzyme cost, based in a total cost of the bioprocess, is relevant. For example, considering the ethanol production from lignocellulosic materials, in an industrial plant that uses enzymatic hydrolysis, the use of cellulases enzymes represents about 18% of the total operational cost. Thus, the improvement of technologies for the fibrolytic enzymes production and application, mainly xylanases and cellulases, in economical bioprocess, represents the key to increase the productivity and the economic viability of the enzymatic route for several applications. These approaches were debated in this work.

Currently, studies are being done to increase the hydrolytic enzymes production by fermentation of agricultural residues (lignocellulolytic materials – LCM) through biotechnological processes resulting in promising yields, and enabling the use of these residues to production costs decrease. There are some microorganism species used in fibrolytic enzymes production, but *Trichoderma* and *Aspergillus* species are the main ones. A variety of agricultural residues can also be used to produce enzymes responsible for degradation of their cell wall since these cultures simulate the natural growth of the fungi and bacteria that degrades the LCM in the environment. The use of LCM by microorganisms is related to the production of cellular proteins, enzymes, organic acids, important secondary metabolites, and also prebiotic oligosaccharides. Pretreatments in these materials also can be used to obtain a bioprocess yields improvement since their objective is to decrease the LCM recalcitrance.

Sugarcane straw is a new residue that is accumulating in the Brazilian fields due to the mechanical harvesting is been applied. The straw composition seems to the other residue from sugarcane crop which has been studied for a long time, sugarcane bagasse. The bagasse is now known as feedstock for the production of several samples of value added.

In this work, sugarcane straw was used as the main LCM substrate and give some products, as xylanases, xylan, that were used to kraft pulp biobleaching, and xylooligosaccharides (XOS) production respectively. XOS were used to fed probiotic microorganisms and fibrolytic yeast, as demonstrated in the Fig. 1:

Fig. 1 - Flowchart with the main processes developed in this work beginning with the residue sugarcane straw.



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GENERAL CONCLUSIONS

The integrated bioprocesses used in this work using sugarcane straw show different manners to reutilize this waste in some biotechnological routes. In Chapter 1 was present a review about fibrolytic enzymes, including its production and applications, that were important to plan our work. Besides that, a comparison between the widely used sugarcane bagasse and sugarcane straw was performed to prove the former can be replaced for the second and the importance to promote strategies to use this abundant residue. In Chapter 2, The optimization of the enzyme production selecting straw as the main carbon source to produce xylanase and cellulase fungal enzymes was performed with high hydrolytic activity, as well the *T. reesei* QM9414 as the best producer. The biochemical characterization was also important to us to plan and execute easily the next steps of enzyme application. In Chapter 3, the crude extract, mainly constituted by xylanases, shows specific activity on hemicellulose from kraft pulp, being easily the lignin removal from pulp after cooking, and consequently almost 10% of chlorine dioxide could be saved in the chemical step of bleaching. The enzyme action could be seen in SEM images. In Chapter 4, the xylan was extract from sugarcane straw and hydrolyzed by two different processes, acid and enzymatic, to produce XOS. The enzymatic route was more interesting due to the good results obtained that were very similar to the acid one and no chemical and neutralization is necessary, despite the need a longer treatment period. Besides that, no furfural was detected in this hydrolyzed. Probiotic bacteria and a yeast was feed by the sugars (xylose and XOS) produced by enzymatic route and these microorganisms tested were capable to grow on enzymatic xylan hydrolisate. The probiotic bacteria *Bifidobacterium longum* BL-05, *B. breve* BB-03, *Lactobacillus brevis* ATCC 367 and *L. acidophilus* ATCC 4356 and the yeast *W. onychis* were able to grow in the enzymatic hydrolysate containing xylose and XOS.

SUGGESTIONS FOR FUTURE RESEARCHES

Based on our results we can considerate the following actions in the future:

- Scale-up the enzymatic production to more than 1.5 L;
- Scale-up the Kraft pulp biobleaching in a pilot scale with the study of economic viability
- Improvement of the xylan extraction, using other chemicals and physical processes, to improve the yields;
- Optimization the xylan enzymatic hydrolysis conditions aiming to produce more XOS with different DP and less xylose and separation of XOS and Xylose;
- Use the cellulose from xylan extraction for the biotechnological processes aiming the integrated bioprocess and biorefinery purpose;
- Study the purification step of the xylose and XOS hydrolysate produced by acid hydrolysis to remove furfural and the evaluation of the growth of probiotic microorganism