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CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA VEGETAL

NELCIELE CAVALIERI DE ALENCAR GUIMARÃES

Efeito das xilanases de *Aspergillus japonicus* var *aculeatus*, *Aspergillus niger* e *Aspergillus flavus* no processo de biobranqueamento e produção enzimática usando resíduos agroindustriais como substrato

Orientação: Drª Maria Rita Marques
Co-orientação: Drª Giovana Cristina Giannesi
Drª Maria de Lourdes T. de M. Polizeli

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Dissertação apresentada como um dos requisitos para obtenção do grau de Mestre em Biologia Vegetal junto ao Departamento de Biologia do Centro de Ciências Biológicas e da Saúde.

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SUMÁRIO

DEDICATÓRIA

Eu dedico este trabalho a Deus, que é o meu pai amoroso, meu guia, e a força que me motiva, pois o senhor é o meu pastor e nada me faltará.

À minha mãezinha, Nossa Senhora do Perpétuo Socorro, que sempre está cuidando de mim nos bons e maus momentos, e intercedendo junto a Jesus Cristo, seu filho, e ao Pai em todos os pedidos que lhe faço.

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INTRODUÇÃO GERAL

RESUMO

Este estudo descreve a produção de xilanases por *A. japonicus* var *aculeatus*, *A. niger* e *A. flavus* a partir de resíduos agroindustriais como fonte de carbono e aplicação destas enzimas no biobranqueamento da polpa de celulose. A melhor fonte de carbono para produção de xilanase por *A. japonicus* var *aculeatus*, *A. niger* e *A. flavus* foi o farelo de trigo (11, 12,76 e 8 U/mg de proteína, respectivamente). Observamos que a xilanase foi induzida similarmente quando o *A. japonicus* foi crescido em xilana 0,5% (10,3 U/mL) e em uma mistura de farelo de trigo e xilana (10 U/mL). A xilanase produzida por *A. niger* foi entre 18 e 21% maior em farelo de trigo 1% do que em xilana, o que significa que este pode substituir a xilana eficientemente. O pH e a temperatura ótimos da xilanase de *A. japonicus* corresponderam a 5,0 e 55ºC, respectivamente. No teste de termoestabilidade a xilanase produzida por *A. japonicus* se manteve estável a 45 e 50ºC, retendo 64% da atividade inicial durante 1 hora de incubação a 45ºC e teve uma meia-vida de 1 hora a 50ºC. Já as xilanases produzidas por *A. niger* e *A. flavus* mantiveram-se totalmente estáveis a 45ºC. A 50ºC ambas as xilanases foram estáveis nos primeiros 20 minutos, onde a xilanase de *A. niger* ainda reteve 85,72% de atividade com 2 horas de incubação, enquanto a de *A. flavus* teve uma meia-vida de mais de 75 minutos. A 55ºC, a xilanase produzida por *A. niger* mostrou-se mais estável que a do *A. flavus* apresentando uma meia-vida de aproximadamente 45 minutos. A xilanase de *A. japonicus* apresentou uma atividade 22% mais alta com glicerol quando comparada ao controle (sem aditivos). Já a atividade xilanolítica de *A. niger* teve um aumento de 20,66% com 2 horas de incubação quando comparada com o controle (sem aditivos), onde foi observada uma meia-vida de aproximadamente 32 minutos para o controle e 60 minutos com glicerol. Já a atividade xilanolítica de *A. flavus* apresentou um aumento de 30% com 2 horas de incubação a 50ºC na presença do glicerol. A xilanase de *A. niger* foi totalmente estável em todos os pHs testados, retendo mais de 95% de atividade durante 1 hora, enquanto a xilanase de *A. flavus* teve uma pequena diminuição de 10% em pH entre 3,0–5,5, mantendo-se estável (100%) em pHs superiores. A alta estabilidade das xilanases de *A. niger* e *A. flavus* sob pH alcalino é muito interessante quando lembramos que o processo de biobranqueamento com xilanase é feito sob condições alcalinas. O melhor resultado do teste de biobranqueamento com a xilanase de *A. japonicus* foi

obtido com 10 U/g de polpa seca durante 3 horas de tratamento, o qual diminuiu 3,9 pontos do número kappa em comparação ao controle (eficiência kappa – 25,2%). Usando a xilanase, a alvura aumentou 2,8, 2,2 e 3,1 pontos. A xilanase de *A. flavus* foi mais efetiva no biobranqueamento quando comparada ao *A. niger*. O número kappa reduziu 5,07 e 3,62 pontos, o que corresponde a 36,32 e 25,93 de eficiência kappa, respectivamente. Os resultados mostraram que as xilanases produzidas por *A. japonicus*, *A. niger* e *A. flavus* têm características promissoras, como boa estabilidade ao pH e a temperatura, para serem industrialmente aplicadas no biobranqueamento da polpa de celulose.

ABSTRACT

This study describes the production of xylanases by *A. japonicus* var *aculeatus*, *A. niger* and *A. flavus* using agroindustrial residues as carbon source and application of theses enzymes on cellulose pulp biobleaching process. The best carbon source for xylanase production by *A. japonicus* var *aculeatus*, *A. niger* and *A. flavus* was the wheat bran (11, 12.76 and 8 U/mg of protein, respectively). We observed that the xylanase was similarly induced when *A. japonicus* was grown on xylan 0.5% (10.3 U/mL) and on a mixture of xylan and wheat bran (10 U/mL). The xylanase produced by *A. niger* was between 18 and 21% higher on wheat bran 1% than on xylan, which means that it can efficiently substitute xylan. The optimum pH and temperature of the *A. japonicus* xylanase corresponded to 5.0 and 55ºC, respectively. In the test of thermostability the xylanase produced by *A. japonicus* remained stable at 45 and 50ºC, retaining 64% of initial activity during 1 hour of incubation at 45°C and had a half-life of 1 hour at 50ºC. The xylanases produced by *A. niger* and *A. flavus* remained totally stable at 45ºC. At 50ºC, both the xylanases were stable in the first twenty minutes, where the xylanase of *A. niger* still retained 85.72% activity with 2 hours of incubation, while of *A. flavus* had a half-life of more than 75 minutes. At 55ºC, the xylanase produced by *A. niger* showed more stable than from *A. flavus* showing a half-life of approximately 45 minutes. The xylanase of *A. japonicus* showed an activity 22% higher with glycerol when compared to control (without additives). The xylanolytic activity of *A. niger* had an increase of 20.66% with 2 hours of incubation when compared with the control (without additives), where it was observed a half-life of approximately 32 minutes for the control and 60 minutes with glycerol. And the xylanolytic activity of *A. flavus* increased 30% with 2 hours of incubation at 50ºC in the presence of glycerol. The xylanase of *A. niger* was totally stable at all the pHs tested, retaining more than 95% activity during 1 hour, while the xylanase from *A. flavus* had a small decrease of 10% at pH between 3.0 – 5.5, maintaining stable (100%) at higher pHs. The high stability of the xylanases of *A. niger* and *A. flavus* under alkaline pH is very interesting when we remember that the biobleaching process with xylanases is done under alkaline conditions. The best result of the test of biobleaching with the *A. japonicus* xylanase was obtained with 10 U/g dry pulp during 3 hours of treatment, that decreased 3.9 points of the kappa number in comparison to the control (kappa efficiency - 25.2%). Using the xylanase, the

brightness improved 2.8, 2.2 and 3.1 points. The xylanase of *A. flavus* was more effective in biobleaching when compared to *A. niger*. The kappa number decreased 5.07 and 3.62 points, corresponding to 36.32 and 25.93 kappa efficiency, respectively. These results showed that the xylanases produced by *A. japonicus*, *A. niger* and *A. flavus* have promising characteristics, such as good stability to pH and temperature, to be industrially applied in cellulose pulp biobleaching.

1. INTRODUÇÃO GERAL

1.1. BIOTECNOLOGIA E PRODUÇÃO DE ENZIMAS

Anualmente, grandes quantidades de resíduos lignocelulósicos (resíduos agrícolas e agroindustriais, como farelo de trigo, bagaço de cana de açúcar, farelo de soja, palha de arroz, sabugo de milho e casca de laranja) são gerados através dos processos de indústrias como cervejarias, indústrias de polpa e papel, têxtil e madeira. O acúmulo destes resíduos leva a deterioração do meio ambiente e perda de recursos, com contribuição significante para o problema da reciclagem da biomassa. Além disso, a maioria desses resíduos é descartada por queima, contribuindo para o aquecimento global (Sepahy et al., 2011; Facchini et al., 2011; Okafor et al., 2007). A existência destes problemas de poluição associado com os resíduos agroindustriais, escassez de lugares para seu depósito, opções de tratamento alto e alta necessidade de salvar recursos valiosos encorajou a utilização e a bioconversão de resíduos em produtos úteis e de alto valor industrial como, biocombustíveis, rações animais, aminoácidos, enzimas e produtos químicos (Chapla et al., 2010). De fato, o uso de resíduos agroindustriais em bioprocessos, é efetivo como substratos alternativos, e podem reduzir problemas de poluição ambiental (Lakshmi et al., 2009). Estes resíduos podem ser utilizados como substrato para a produção de enzimas por microrganismos, devido ao seu elevado teor de proteínas e carboidratos, reduzindo os custos na produção de enzimas de caráter biotecnológico (Kronbauer et al., 2007). Dessa forma, a produção das enzimas de interesse biotecnológico a partir de resíduos agroindustriais é uma alternativa para as indústrias de biotecnologia obter enzimas hidrolíticas e oxidativas com um custo mais barato de produção em relação às enzimas que

estão no mercado, como também uma maneira viável de agregar valor a estes resíduos, diminuindo seu impacto ambiental. As enzimas microbianas são as mais utilizadas devido às facilidades de obtenção destas em relação às outras, pois é possível: obter uma grande população desses organismos, selecionar cepas de alta e rápida produtividade, a recuperação da enzima é mais simples e o fabricante pode monitorar sua produção em todas as fases (Sandrim, 2003).

1.2. COMPONENTES ESTRUTURAIS DA MADEIRA

A biomassa de plantas considerada como “resíduos” é biodegradável e representa uma fonte de carbono alternativa para crescimento de microrganismos ou produção de enzimas. Esta é constituída de carboidratos, sendo os glicanos e as hemiceluloses os polissacarídeos de maior importância. Entre os glicanos, que são homopolissacarídeos constituídos de unidades monoméricas de D-glicopiranoses, o amido (α1→4 e α1→6) e a celulose (β1→4) estão entre os mais abundantes, sendo que a diferença estrutural entre estes dois biopolímeros está no tipo de ligação glicosídica que formam.

Por outro lado, as hemiceluloses são polímeros complexos de homo ou heteropolissacarídeos ramificados, sendo formadas principalmente pelas aldopentoses xilose e arabinose, pelas aldo-hexoses glicose e manose e pelos ácidos metilglucurônico e galacturônico, não possuindo, portanto, nenhuma relação estrutural com a celulose, como mostra a Figura 1 (Ribeiro et al., 2012).

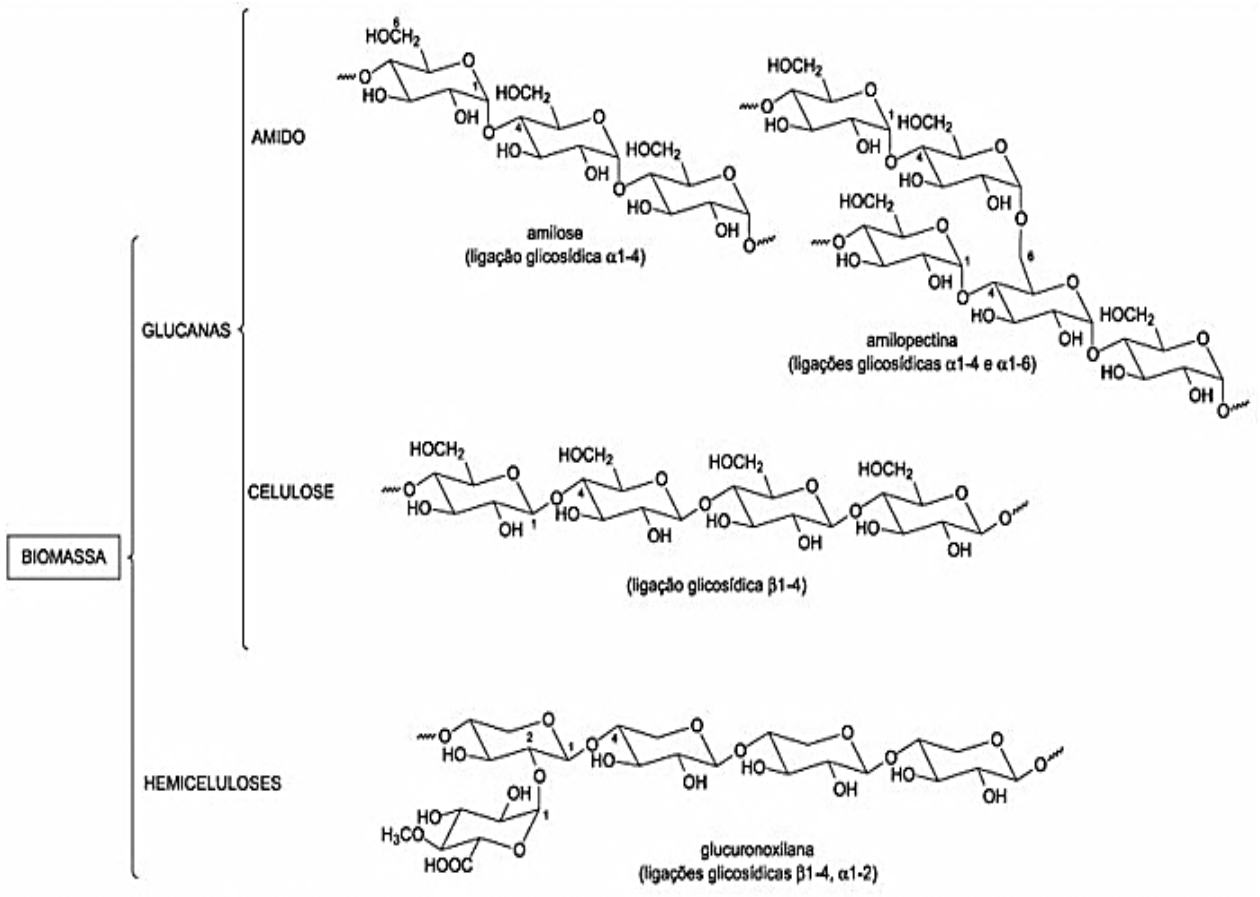

Figura 1. *Representação estrutural parcial dos principais polissacarídeos presentes na biomassa* (Ribeiro et al., 2012).

A maneira como essas substâncias constituintes da madeira estão organizadas formando a parede celular vegetal está ilustrada na Figura 2. Esta é constituída de paredes primárias e secundárias, onde a primária (P) é formada por 10, 20 e 70% de celulose, hemicelulose e lignina, respectivamente, e ocupa de 7 a 14% da parede celular. A parede secundária que se divide em três camadas distintas é constituída por S1, que corresponde de 5 a 11% da parede celular, S2 de 74 a 84% e S3 de 3 a 4%. Estas são classificadas segundo a orientação das fibras de celulose, sendo que a proporção desses componentes em S1 correspondem a 35, 25 e 40%, em S2 a 55, 30 e 15% e em S3 a 55, 40 e 5% de celulose, hemicelulose e lignina,

respectivamente. Na lamela média (LM), cuja função é aderir uma célula à outra formando um tecido, a proporção desses componentes corresponde a 0, 10 e 90% (Polizeli, 2008; Mitchell et al., 1992).

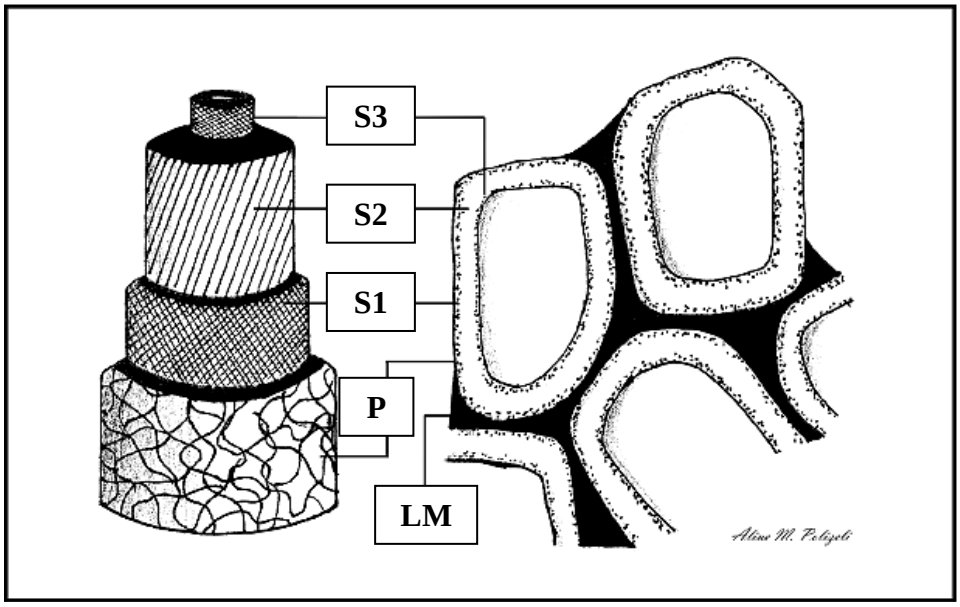

Figura 2. *Esquema básico da estrutura da parede celular de madeiras moles e de madeiras duras. LM - lamela média; P - parede celular primária e camadas da parede celular secundária S1, S2 e S3 (modificado de Polizeli, 2008).*

Nas plantas terrestres, as hemiceluloses, que representam cerca de um terço de todas as fontes de carbono orgânico renovável da Terra, podem ser compostas por uma variedade de monossacarídeos como: D-xilose, D-manose, D-galactose, D-glicose, L-arabinofuranose, ácido 4-*O*-metil-D-glucurônico, ácido galacturônico e seus derivados acetilados ou metilados, sendo classificadas geralmente de acordo com os resíduos de açúcares que as compõem (Sandrim, 2003).

A xilana, principal componente da hemicelulose, é o segundo polissacarídeo mais abundante e renovável da natureza, e está localizada entre

a molécula de lignina e o conjunto de fibras de celulose (Birijlall et al., 2011). Esta constitui cerca de 20-35% do total de peso seco em biomassa tropical (Das et al., 2013), e é composta por resíduos de β-xilopiranosil unidos através de ligação do tipo β-1,4 na cadeia principal que, na natureza, geralmente está associada a outros açúcares, formando glucuronoxilanas, glucuronoarabinoxilanas, glucomananos, arabinogalactanos e galactoglucomananos (Sepahy et al., 2011; Peixoto-Nogueira, 2009).

A xilana é a principal hemicelulose de madeiras provenientes de angiospermas (15 - 30% do peso seco total), sendo menos abundante em madeiras de gimnospermas (7 – 12%). Em madeiras duras (angiospermas), a xilana é formada por pelo menos 70 resíduos de β-xilopiranosil. Cada décimo resíduo de xilose carrega um ácido α-4-*O*-metilglucurônico no C2. Além disso, estas xilanas são altamente acetiladas (70%). A acetilação pode ocorrer tanto no C2, quanto no C3, conferindo a xilana sua parcial solubilidade em água. Por estas razões são denominadas *O*-acetil-4-*O*-metilglucuronoxilanas (Figura 3A). Já em madeira mole (gimnospermas) a xilana é composta por arabino-4-*O*-metilglucuronoxilana (±10%) (Figura 3B), apresentando um conteúdo maior em ácido α-4-*O*-metilglucurônico do que as madeiras duras, ligados no C2 da xilose. Porém, as xilanas de madeira mole não são acetiladas, e no lugar do grupo acetil apresentam um grupo α-L-arabinofuranosil unidos ao C3 da xilose por ligações glicosídicas α-1,3 (Polizeli et al., 2005; Sunna & Antranikian, 1997; Ferreira-Filho, 1994). A abundância e tipos de ligação entre estas substituições variam entre xilanas de diferentes fontes.

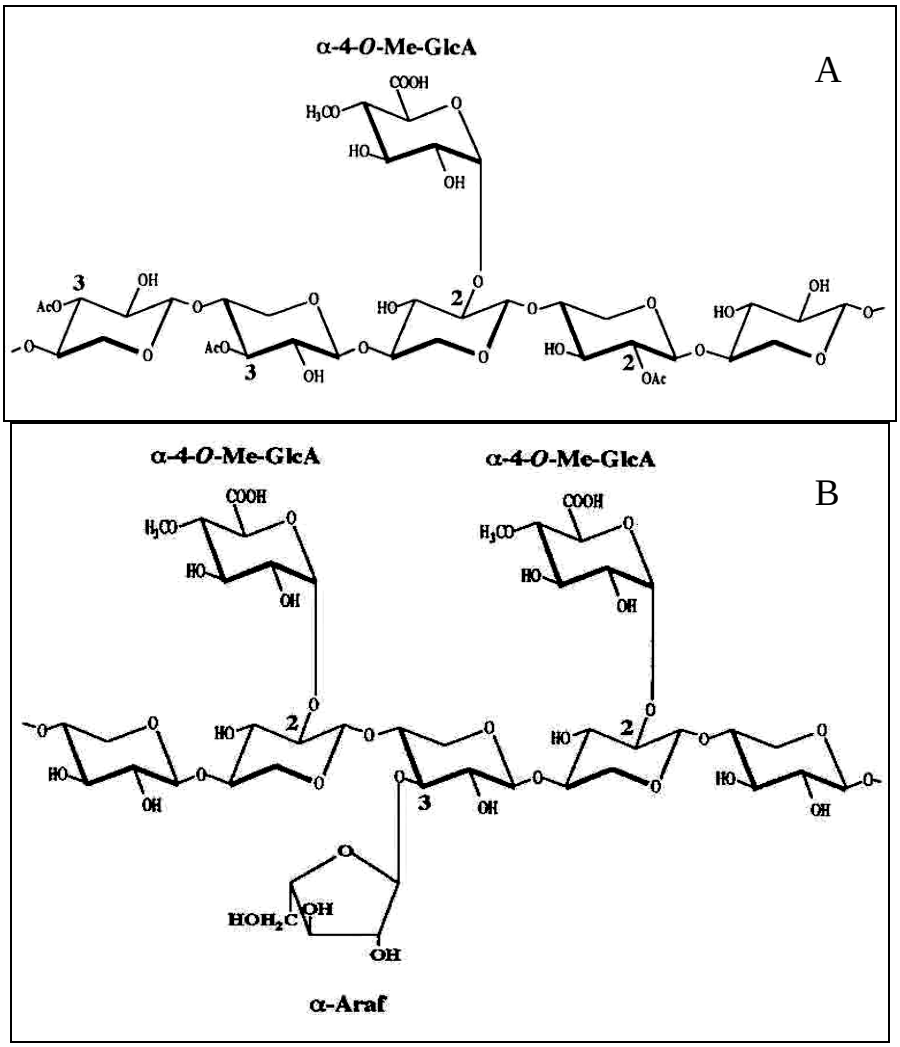

Figura 3 *– Estrutura da O-acetil-4-O-metilglucuronoxilana de madeira dura (A) e da arabino-4-O-metilglucuronoxilana de madeira mole (B). Os números indicam os átomos de carbono aos quais ligam-se os grupos substituintes. Abreviações: Ac – grupo acetil; α-4-O-Me-GlcA – ácido α-4-O-metilglucurônico; α-Araf – α-arabinofuranose (adaptado de Sunna & Antranikian, 1997).*

1.3. DEGRADAÇÃO ENZIMÁTICA DAS XILANAS

Devido a sua heterogeneidade estrutural, a degradação da xilana requer a ação de várias enzimas, ou seja, de um sistema enzimático que se encontra presente em fungos e bactérias principalmente (Peixoto-Nogueira, 2009).

As principais enzimas desse sistema enzimático responsável pela degradação da xilana são as β-1,4-endoxilanases (1,4-β-D-xilanaxilohidrolase; EC 3.2.1.8) e as β-xilosidases (β-D-xilosídeoxilohidrolase; EC 3.2.1.37) (Figura 4). As endoxilanases hidrolisam ligações glicosídicas internas da cadeia principal da xilana produzindo xilooligossacarídeos, resultando em uma diminuição no grau de polimerização do substrato. Essa clivagem não ocorre ao acaso, uma vez que as ligações a serem hidrolisadas dependem da natureza do substrato, isto é, do comprimento, do grau de ramificação e da presença de substituintes (Peixoto-Nogueira, 2009; Polizeli et al., 2005; Li et al., 2000). E as β-xilosidases, que são exoglicosidases, clivam estes xilooligossacarídeos pequenos e xilobiose a partir da extremidade não redutora liberando xilose (Wakiyama et al., 2010). Mas também podem estar presentes α-arabinofuranosidases, que removem os resíduos de L-arabinose substituídos no C3 das unidades de xilose (Kaneko et al., 1993); α-glucuronidases (EC 3.2.1), que hidrolisam as ligações α-1,2 entre ácido glucurônico e resíduos de xilose na glucuronoxilana (Chávez et al., 2006); acetil-xilanaesterases (EC 3.1.1.6), que removem os substituintes *O*-acetil a partir da posição C2 e/ou C3 dos resíduos de xilose na acetil xilana (Chávez et al., 2006; Caufrier et al., 2003) e algumas esterases, como o ácido ferúlico esterase (EC 3.1.1) e o ácido p-coumárico esterase (EC 3.1.1) que clivam ligações éster na xilana, respectivamente, entre as cadeias laterais de arabinose e do ácido ferúlico, e entre arabinose e ácido p-coumárico (Chávez et al., 2006; Crepin et al., 2004; Williamson et al., 1998).

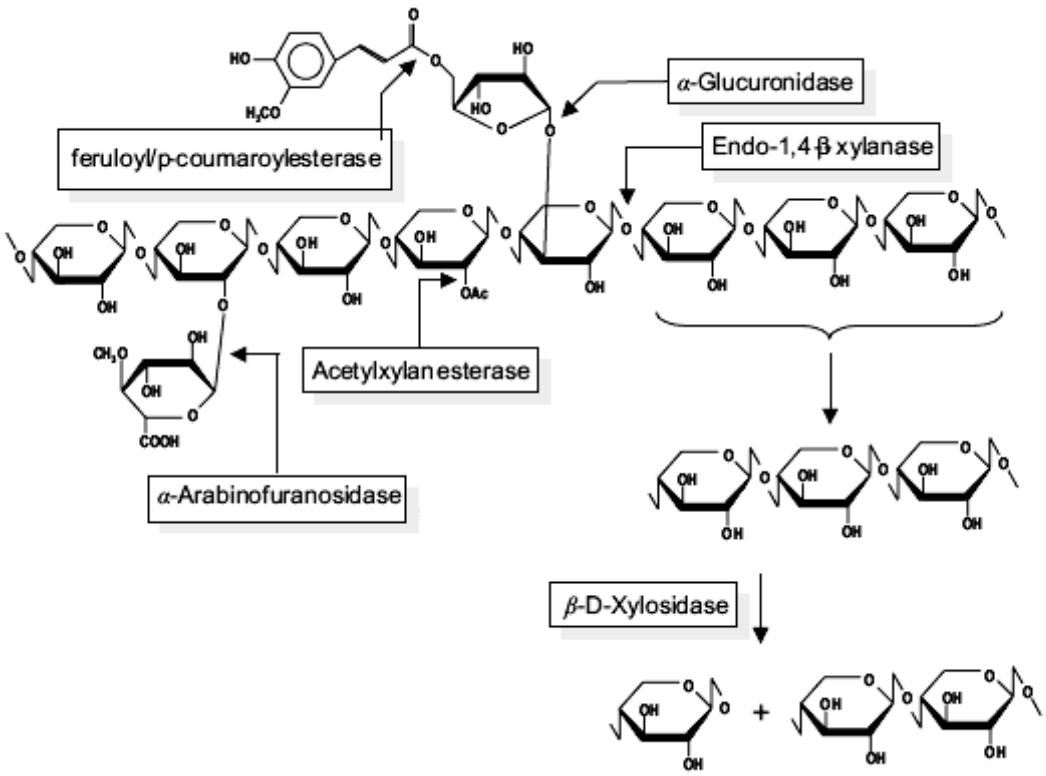

Figura 4. *Representação esquemática de uma molécula de xilana e das enzimas do sistema xilanolítico (Polizeli, 2008).*

1.4. APLICAÇÕES INDUSTRIAIS DAS XILANASES

O grande interesse no estudo de enzimas está na sua possibilidade de aplicação em diversos setores da indústria melhorando procedimentos e produtos já existentes, bem como no estabelecimento de novos processos (Peixoto-Nogueira, 2009). Estas estão envolvidas na bioconversão da xilana, altamente presente em resíduos agrícolas, em xilose, o qual age como substrato na obtenção de etanol através de processos de fermentação (Chandrakant & Bisaria, 2000) e em xilitol, utilizado como adoçante em vários tipos de alimentos (Parajó et al., 1998). Além disso, o uso de xilanases tem se mostrado bastante favorável, pois uma variedade de microrganismos, incluindo

bactérias, leveduras e fungos filamentosos são produtores de xilanases, sendo capazes de degradar e utilizar celulose e hemicelulose como fonte de carbono e energia, nos quais os fungos se destacam com a maior produção (Lee et al., 2009; Fengxia et al., 2008). Dentre os fungos produtores de xilanases a partir de resíduos agroindustriais, tem-se *Aspergillus terreus* (Sorgatto et al., 2012); *Aspergillus niger* (Khonzue et al., 2011; Dhillon et al., 2011; Farinas et al., 2010; Pal & Khanum, 2010), *Aspergillus terricola* Marchal (Michelin et al., 2010) e *Aspergillus foetidus* (Chapla et al., 2010) produtores de xilanase a partir de farelo de trigo; *Penicillium echinulatum* (Camassola & Dillon, 2008), *Penicillium janthinellum* (Meshram et al., 2008) e *Aspergillus caespitosus* (Sandrim et al., 2005) em bagaço de cana de açúcar; *Aspergillus terreus* (Ghanem et al., 2000), *Paecilomyces thermophila* (Li et al., 2006), *Aspergillus* sp e *Thermomyces lanuginosus* (Puchart & Biely, 2008) em sabugo de milho e trigo. Porém, xilanases são produzidas principalmente por *Aspergillus* e *Trichoderma* sp em escala industrial (Carapito et al., 2009; Betini et al., 2009; Fengxia et al., 2008). Mas, as xilanases produzidas por *Aspergillus flavus* tem sido pouco relatadas na literatura.

Estas enzimas têm sido amplamente empregadas em várias indústrias, para a produção de hidrolisados de resíduos agroindustriais, processamento de alimentos, aumento da digestibilidade da ração animal (Facchini et al., 2011; Tapingkae et al., 2008), clarificação de sucos e vinhos (Lee et al., 2009; Fengxia et al., 2008; Sandrim et al., 2005) e na indústria têxtil e farmacêutica (Ustinov et al., 2008). Na indústria alimentícia, como por exemplo, na panificação, as xilanases são adicionadas ao pão para aumentar o seu volume específico, determinando a textura do miolo e seu sabor final (Shah et al.,

2006). Já na fabricação de cerveja, a parede celular da cevada é hidrolisada permitindo a quebra do amido, porém há liberação de longas cadeias de arabinoxilanas, que aumentam a viscosidade podendo deixar a cerveja turva. Assim, as xilanases auxiliam na solubilização das arabinoxilanas aos oligossacarídeos menores, diminuindo sua viscosidade e consequentemente eliminando a turbidez da cerveja (Rizzatti et al., 2004). Mas a principal aplicação das xilanases atualmente é no processo de biobranqueamento da polpa de celulose (Das et al., 2013; Peixoto-Nogueira et al., 2009; Betini et al., 2009).

1.5. APLICAÇÃO BIOTECNOLÓGICA NA INDÚSTRIA DE PAPEL

Devido à crescente demanda por papel, a indústria de papel e celulose está crescendo rapidamente e se tornando um dos piores poluidores em termos ambientais (Savitha et al., 2007). O uso de tecnologia favorável ao ambiente é realmente preferível, em qualquer processo industrial, por isso, devido à grande preocupação sobre os riscos ambientais de substâncias químicas tóxicas utilizadas nessas indústrias, os métodos alternativos de branqueamento de papel têm sido sugeridos (Yeasmin et al., 2011).

A fabricação do papel inicia-se com a lavagem e o descascamento das toras de madeira. Segue-se a picagem do material em cavacos visando facilitar a difusão dos reagentes utilizados no tratamento da polpa em um processo denominado de polpação. Este processo de polpação tem como objetivo facilitar a separação das fibras e melhorar suas propriedades para a fabricação do papel. Esta polpação pode ser realizada por meio de um processo químico

no qual a maior parte da lignina é retirada da madeira, mas, com a utilização deste processo, somente 40 –50% da massa total inicial da madeira é aproveitada. Há outros processos mecânicos de polpação nos quais ocorre mínima remoção dos componentes da madeira, levando ao seu aproveitamento quase que total (Peixoto-Nogueira, 2009).

No Brasil, o processo de polpação mais utilizado é conhecido como “Processo kraft”, o qual se inicia com o cozimento de cavacos de madeira em uma solução contendo hidróxido de sódio (NaOH) e sulfeto de sódio (Na_2S) dentro de um digestor em condições elevadas de temperatura e pressão. Nesta etapa, aproximadamente 90 – 95% da lignina, principal responsável pela cor escura do papel, é solubilizada no licor de cozimento, fragmentando-a em substâncias de baixa massa molar que vão solubilizar em pH alcalino e ser removidas por meio de inúmeras lavagens com oxigênio (pré-branqueamento), processo que tem o objetivo de reduzir os custos de outros reagentes empregados no processo de branqueamento (Peixoto-Nogueira, 2009; Polizeli et al., 2005; Sandrim, 2003). A polpa pré-branqueada tem uma cor amarela pálida e ainda não é adequada para a produção de papel de boa qualidade para impressão e escrita (Polizeli et al., 2005). O passo seguinte é o branqueamento, o qual gera uma polpa branca o suficiente para produzir papel. Nesta etapa, são utilizados cloro (Cl_2), dióxido de cloro (ClO_2), peróxido de hidrogênio (H_2O_2), oxigênio (O_2) e hidróxido de sódio (NaOH), os quais agem degradando os anéis da lignina, acarretando na sua solubilização durante o processo(Gellerstedt, 2001). Um resumo do processo de produção da polpa de celulose está representado na Figura 5.

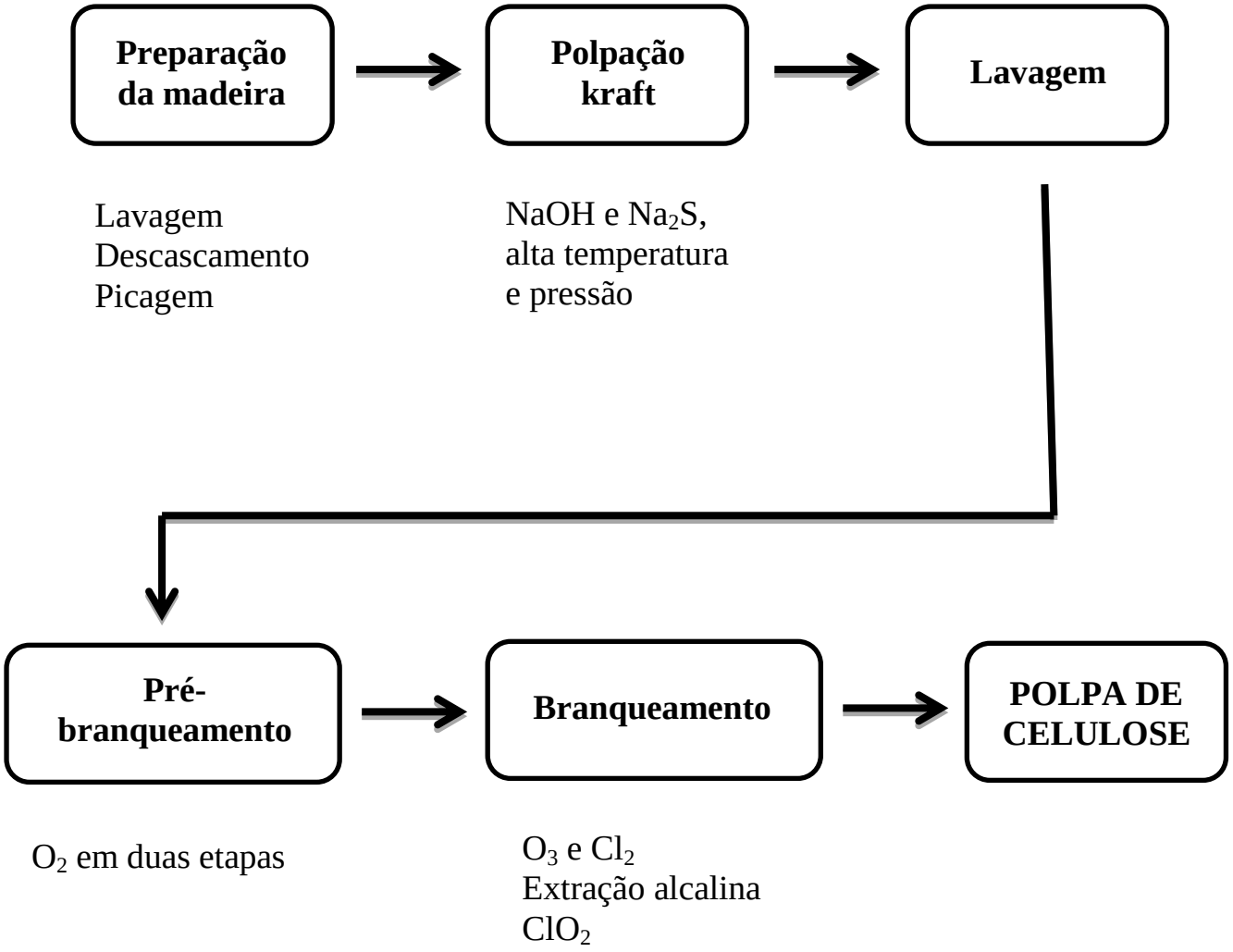

Figura 5. *Esquema representativo de um processo de produção de polpa de celulose (adaptado de Peixoto-Nogueira, 2009).*

Os processos acima descritos geram quantidades significativas de poluentes. É neste contexto que atualmente, a utilização de xilanases para biobranqueamento da polpa é reconhecida como um processo baseado em biotecnologia economicamente viável nas indústrias de papel e celulose. O pré-branqueamento com xilanases faz exatamente essa função de reduzir a necessidade de branqueadores químicos tóxicos (cloro), colaborando com a redução de compostos orgânicos clorados liberados nos efluentes das instalações de branqueamento, redução do número kappa (teor de lignina

residual na polpa), consequentemente aumentando o brilho da polpa de celulose e é, portanto, ambiental e economicamente vantajoso (Khonzue et al., 2011; Medeiros et al., 2007). Este processo baseia-se na especificidade única de hemicelulases, particularmente enzimas xilanolíticas, atacando os componentes de hemicelulose na polpa (Khonzue et al., 2011). Xilanases e outras hemicelulases são utilizadas para modificar a estrutura da xilana e glucomanano, em fibras de celulose, a fim de melhorar a eficiência de deslignificação química. β-endoxilanases causam hidrólise da cadeia principal daxilana na polpa, reduzindo o grau de polimerização do substrato, e são utilizadas principalmente para a remoção do complexo de lignina-carboidrato (LCC) gerado no processo kraft, agindo como uma barreira física contra a entrada de produtos químicos de branqueamento. A remoção do LCC, assim, facilita a extração eficiente de lignina de alta massa molecular da celulose (Khonzue et al, 2011; Peixoto-Nogueira et al., 2009).

Para aplicação no biobranqueamento, a xilanase deve ser termoestável, tolerante a alcalinos e estável sobre a polpação kraft, e suas diversas propriedades, tais como baixo peso molecular e padrão de ação específica devem atender as exigências do processo de polpação. Além disso, a fim de evitar danos para a polpa de celulose, as preparações enzimáticas devem estar isentas de atividade de celulase (Yeasmin et al., 2011).

2. OBJETIVOS

2. OBJETIVOS

2.1. OBJETIVO GERAL

O objetivo deste trabalho foi caracterizar as xilanases extracelulares produzidas por *Aspergillus japonicus var aculeatus*, *A. niger* e *A. flavus* obtidas a partir de fermentação submersa e aplicação destas enzimas no biobranqueamento da polpa da celulose.

2.2. OBJETIVOS ESPECÍFICOS

- Produção de xilanases extracelulares usando diferentes fontes de carbono (farelo de trigo, sabugo de milho, bagaço de cana, farelo de arroz, entre outros);
- Caracterização das enzimas (pH, temperatura, termoestabilidade, termoestabilidade com protetores, estabilidade ao pH);
- Aplicação das enzimas no biobranqueamento e na alvura da polpa de celulose.

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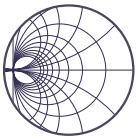
Anexos

(ARTIGO I)

Guimarães et al., J Biocatal Biotransformation 2013, 2:1
http://dx.doi.org/10.4172/2324-9099.1000105



Journal of Biocatalysis & Biotransformation

Research Article

A SCITECHNOL JOURNAL

Xylanase Production from *Aspergillus japonicus* var *aculeatus*: Production using Agroindustrial Residues and Biobleaching Effect on Pulp

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Abstract

This study describes a xylanase produced by *A. japonicus* var *aculeatus,* and evaluated the suitability of this protein for cellulose pulp biobleaching process. The best production of xylanase was achieved with wheat bran (AE=11 U/mg of protein). We observed that the xylanase was induced similarly, when the *A. japonicus* was grown on xylan 0.5% (10.3 U/ml), and on a mixture of wheat bran and xylan (10 U/ml). The optimum xylanase pH and temperature corresponded to 5.0 and 55°C, respectively. The xylanase remained stable at 45 and 50°C, retaining 64% of the initial activity during 1 hour of incubation at 45°C, and had its half-life corresponded to 1 hour at 50°C. The xylanase activity increased 22% with glycerol, when compared to the control (without additives), incubated during 1 hour at 50°C. The best result of biobleaching was obtained with 10 U/g dry pulp for 3 hours of treatment, that decreased 3.9 points of the kappa number, in comparison to the control (kappa efficiency-25.2%). Using the xylanase, the brightness improved 2.8, 2.2 and 3.1 points. The results showed that the xylanase produced by *A. japonicus* has promising characteristics, to be industrially applied on the biobleaching of cellulose pulp.

Keywords

Xylanase; *Aspergillus japonicus* var *aculeatus*; Agroindustrial residues; Biobleaching

Introduction

Due to the ever-increasing demand for paper, the paper pulp industry is rapidly growing and becoming one of the worst offenders in environmental terms [1]. The use of eco-friendly technology is actually preferred in any industrial process; therefore, due to severe concern over the environmental hazards of toxic chemicals used in pulp and paper industries, alternative methods for bleaching of pulp have been suggested [2].

At present, the use of xylanases for biobleaching of pulp is recognized as an economically feasible biotechnology-based process in the pulp and paper industries. Pre-bleaching with xylanases does exactly this function of reducing the need for toxic bleaching chemicals, and is thus environmentally and economically advantageous [3].

An alternative approach in eliminating chlorine in bleaching, reducing chlorinated organic compounds bleach plant effluents, reducing the kappa number (residual lignin content in the pulp) and increasing the brightness of the pulp, is the use of xylanases in prebleaching of cellulose pulp [4]. It is based on the unique specificity of hemicellulases, particularly xylanolytic enzymes, in attacking the hemicellulose component in pulp [3].

Xylanases and other hemicellulases are used to modify the structure of xylan and glucomannan in pulp fibers, in order to enhance chemical delignification efficiency. Endo-β-xylanases cause hydrolysis of the main chain of xylan in pulp, reducing the degree of polymerization of substrate, and are used primarily for the removal of the lignin-carbohydrate complex (LCC), generated in the kraft process, acting as a physical barrier against the entry of bleaching chemicals. LCC removal, thus, facilitates the efficient extraction of high molecular mass lignin from pulp [5].

For biobleaching applications, the candidate xylanase should be thermostable, alkali tolerant and stable on kraft pulping, and its various properties, such as low molecular weight and specific action pattern, must suit the pulping process requirements. Moreover, to avoid damage to cellulose pulp, enzyme preparations should be free from cellulase activity [6].

The aim of the present study was to describe the extracellular xylanase produced by *Aspergillus japonicus* var *aculeatus,* and test its suitability for cellulose pulp biobleaching.

Materials and Methods

Microorganism

Aspergillus japonicus var *aculeatus* strain was isolated by us from soil samples, identified in the Federal University of Pernambuco–UFPE (PE, Brazil), and deposited in our laboratory fungi collection. Stock cultures were propagated at 30°C on slants of solid potato dextrose agar (PDA) medium, stored at 4°C.

Xylanase production and enzyme extraction

Spores were inoculated into 125 ml Erlenmeyer flasks, containing 25 ml medium [7], using 1% (w/v) of the desired carbon sources (1% of xylan or agroindustrial residues, such as rice straw, sugarcane bagasse, wheat bran or corncob). The cultures were incubated under orbital agitation (100 rpm), or stationary conditions, at 30°C, during different periods (24-168 hours). The medium was subsequently vacuum-filtered using filter paper (Whatman nº1), and the crude filtrate was used for the study of the extracellular enzyme.

Enzymatic assays and protein determination

The reaction mixture consisted in 500 µl of citrate-phosphate buffer [8], pH 5.0, containing 1.0% (w/v) of xylan, and 500 µl of enzymatic extract, appropriately diluted. The samples were incubated at 55°C to determine xylanase activity. The amount of reducing sugar released was determined using the 3,5-dinitrosalicylic acid (DNS)

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method, employing xylose (Sigma) as the standard. One unit of enzyme activity was defined as the amount of enzyme which releases 1 µmol of reducing sugar per minute, under assay condition. Specific activities were expressed as U/mg of protein. Protein concentrations were determined by the Lowry method [9], using bovine serum albumin (BSA), as the standard.

Effects of pH and temperature

The effect of pH and temperature on xylanase activity was analyzed using crude filtrate from *A. japonicus*. The suitable pH value for xylanase activity was assayed using McIlvaine buffer, in the pH range 3.5-8.0, at 55°C. The assays of temperature were performed in the same buffer pH 5.0, incubated at different temperatures (35-60°C). To determine thermal stability, the enzyme was incubated between 45 and 60°C for different durations (5 to 60 min), and the assays were performed using McIvaine buffer pH 5.0 at 55°C.

Biobleaching

The amounts of enzyme used for this treatment was 10, 20 or 40 units of enzyme per gram of dried cellulose pulp from *Eucalyptus grandis*. The treatments were performed during different periods (1-24 hours). All calculations and procedures were determined, according to the standard methods of Technical Association of the Pulp and Paper Industry [10]. The consistency was determined on a percent dry weight basis. The volume of enzyme or distilled water was added, until it reached a 10% pulp consistency. Crude xylanase extract from *A. japonicus* was added to the treated pulp, and the control was prepared by adding distilled water, instead of enzyme. The samples were incubated inside sealed polyethylene bags at 50°C for 24 hours, and after that, the treated cellulose pulps were filtered on a Büchner funnel, rinsed with 200 ml of distilled water, and used for determination of kappa number and brightness parameters. The liberation of aromatic compounds was monitored by absorbance values at 237 nm.

Reproducibility of results

All results are expressed as the means of at least three independent experiments.

Results and Discussion

Time course for xylanase production

Nutritional and environmental factors, such as the type of carbon source, time course, use of agitation (or not), temperature and pH, may affect enzyme synthesis and production by fungi [11]. To evaluate the growth time and the effect of stationary and agitated conditions in the production of extracellular xylanase by the fungus *A. japonicus*, it was grown in SR medium [7], with 1% wheat bran for 168 hours. It was observed that the production of xylanase was better in medium under stationary condition than in the medium under agitation (Figure 1). Under stationary conditions, there was a progressive increase of xylanase production in the period, 24-96 hours of cultivation (the period in which there was maximum production), followed by a reduction (45%), and stabilization in the production of the enzyme. Probably, the reduction in xylanase yield was due to the depletion of available nutrients to microorganism, or due to the proteolysis [12]. Under agitated condition, there was a xylanolytic production between 24 and 96 hours, with maximum peak at 96 hours, with a subsequent decrease, but these values are much lower than those produced under stationary condition.

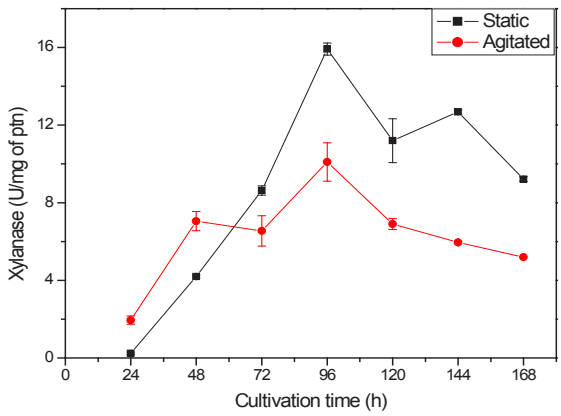

Figure 1: Growth time of extracellular xylanase production by *A. japonicus* (24-168 hours), under stationary and agitated conditions (100 rpm).

Aspergillus foetidus MTCC 4898 grown on solid fermentation using wheat bran as carbon source, also had maximal xylanase production between 24 and 96 hours of cultivation [13]. A similar result was observed at work using the fungus *A. casielus,* with brewery industrial residue as carbon source [14]. *A. fumigatus* RP04 [5] and *A. niger* [15], also had maximal xylanase production with 96 hours of cultivation.

Xylanase production using different agroindustrial residues and substrates

In order to induce xylanase synthesis from microbial sources, agricultural residues such as wheat bran, oat flakes, corn flakes, corncob, rice straw, sugarcane bagasse and others, can be used. The use of agricultural residues as alternative carbon sources reduces the production costs and the price of the final product [16].

The xylanase production by *A. japonicus* was evaluated using different alternative carbon sources. The best production of xylanase was achieved with wheat bran (AE=11 U/mg of protein), followed by soybean bran (AE=7 U/mg of protein), and sugarcane bagasse (AE=5.6 U/mg of protein) (Table 1).

In literature, we found several studies using industrial and agroindustrial wastes as inducing sources for xylanase production [11,12,17]. Among the fungi producers of xylanase from agroindustrial wastes, has *Aspergillus terreus* [11], *Aspergillus terricola* Marchal [17], *Aspergillus foetidus* MTCC 4898 [13] and *Aspergillus ficuum* AF-98 [18], producing xylanase from wheat bran. And in work with *Aspergillus japonicus* C03, the xylanase production was better induced with soybean bran [19].

After finding that the wheat bran was the best substrate inducer of xylanase production by *A. japonicus*, the influence of mixtures of substrates was evaluated (Table 1). We can note that the xylanase was similarly induced, when the *A. japonicus* was grown in xylan, and in the medium containing a mixture of wheat bran and xylan. The enzymatic activity produced in the presence of 0.5% birchwood xylan or oat spelt xylan (10.3 and 9 U/ml, respectively), was almost the same of the mixtures of xylans with wheat bran (10 and 10.6 U/ml, respectively), and of the test only with 0.5% of wheat bran (8.7 U/ml), demonstrating that the use of alternative carbon sources shown viable, as well as favoring the cheaper of the cost of enzyme

production, since the commercial xylan is extremely expensive for widespread use.

There are several studies using different combinations of industrial and agroindustrial wastes as carbon sources for xylanase production in literature [3,19]. Facchini et al. [19] found that a mixture of soybean meal and crushed corncob increased xylanolytic activity by 8.5%. In work using the fungus *A. niger*, *A. niveus* and *A. ochraceus,* and some agroindustrial residues, wheat bran mixed with corncob increased production by about 20% for *A. ochraceus* and *A. niger*, however, not improved the production of xylanase by *A. niveus* [16]. In studies with *A. fumigatus* RP04 and *A. niveus* RP05 was possible to prove that the production of xylanase by *A. fumigatus* was between 5 and 6% higher with corncob meal and wheat meal, as compared with media containing birchwood xylan as carbon source [5].

A higher xylanase production using wheat bran may possibly be due to its low lignin content and higher protein content, compared to other substrates (14.87% protein) [13]. So to optimize the xylanolytic activity, tests were performed with different concentrations of wheat bran, as shown in table 2. The results showed that with increasing of wheat bran concentrations, there was a gradual reduction of xylanolytic activity. At a concentration of 0.5 to 1.5%, there was a high xylanase production as compared to higher values of wheat bran, showing that a small amount of wheat bran is sufficient for a good production of xylanase, consequently reducing production costs. Presumably, 4% inoculum level was so high, that the nutrients were consumed faster, and it overall resulted in a lower enzyme yield [12]. There are few studies of this test with fungi, but in work with *Bacillus mojavensis*, maximum xylanase activity was observed in the presence of 2% oat bran as substrate inducer of the enzyme, and this also had

Table 1: Effect of carbon source on xylanase production by *A. japonicas*.

Carbon Source	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg of protein)
Glucose	1	1	1 (± 0.25)
Rice straw	1	1	1 (± 0.21)
Corncob	3	1	3 (± 0.25)
Sugarcane bagasse	9	2	5 (± 0.21)
Soybean bran	7	1	7 (± 0.26)
Avicel 1%	0.3	0.5	0.6 (± 0.3)
Avicel 0.5%	0.5	0.5	1 (± 0.26)
Wheat bran 1%	11	1.2	9 (± 0.25)
Wheat bran 0.5%	8.7	0.8	11 (± 0.2)
Xylan (oat spelt) 1%	10.3	0.7	15 (± 0.2)
Xylan (oat spelt) 0.5%	9	0.5	18 (± 0.3)
Xylan (birchwood) 1%	10.8	0.7	15 (± 0.2)
Xylan (birchwood) 0.5%	10.3	0.6	17 (± 0.3)
Xylan (oat spelt) 0.5%+Wheat bran 0.5%	10.6	0.9	12 (± 0.3)
Xylan (birchwood) 0.5%+Wheat bran 0.5%	10	1	10 (± 0.2)
Avicel 0.5%+Wheat bran 0.5%	6.2	0.9	7 (± 0.27)

The fungus *A. japonicus* was grown in Erlenmeyer of 125 ml containing SR liquid medium [7], and different carbon sources, which were incubated for 96 h at 30°C in stationary condition. The temperature used for enzyme assay was 55°C.

Table 2: Effect of different concentrations of wheat bran on xylanase production by *A. japonicas*.

[Wheat bran]	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg of protein)
0.5%	8.7	0.8	10.9 (± 0.2)
1.0%	11	1.2	9.2 (± 0.25)
1.5%	6.8	1.0	6.8 (± 0.29)
2.5%	5.6	1.1	5.1 (± 0.31)
4.0%	3.5	0.9	3.9 (± 0.30)
8.0%	3.6	1.2	3.0 (± 0.25)

The fungus *A. japonicus* was grown in Erlenmeyer of 125 ml containing SR liquid medium [7], and different concentrations of wheat bran (0-8.0%), which were incubated for 96 h at 30°C in stationary condition. The temperature used for enzyme assay was 55°C.

a decreased production in higher concentrations [12]. In studies with *Pseudomonas* sp. WLUN024, the yield of xylanase increased greatly with increasing concentrations of wheat bran [20], as well as work with *Streptomyces cyaneus* SN32 [21].

Effect of pH on xylanase activity

The extracellular xylanolytic activity produced by *A. japonicus* showed stable enzymatic activity in the pH range between 4.0 and 6.5, reaching maximum activity at pH 5.0 (Figure 2). These results were similar to other studies, where most of the fungal species, *Aspergillus* sp. showed maximum xylanolytic activity at pH ranging from 4.0 to 6.0, as *A. japonicus* (5.0) [22], *A. ochraceus* (5.0) [17]; *A. fumigatus* RP04 (5.0-5.5), and *A. niveus* RP05 (4.5-5.0) [5]; *A. ochraceus* (5.0), *A. niger* (5.5-6.0) and *A. niveus* (5.0-5.5) [16].

Effect of temperature on xylanase activity and stability

The optimum temperature for *A. japonicus* was evaluated in the range 35-60°C, where the xylanolytic activity had a gradual increase with higher temperatures, and had maximum activity at 55°C, and after a drop of 20% at 60°C (Figure 3). This result agrees with the fact that the optimum temperature for xylanase produced by most fungi is in the range of 40-60°C [23]. In the literature, it cites other microorganisms with similar optimum temperatures such as 55°C for xylanolytic activity of *A. terreus* and 50°C to *A. aculeatus* and *A. sydowii* [24], and *A. casielus* [14]. Studies with *A. niger*, *A. niveus* and *A. ochraceus* shows optimum temperature range 55-65°C [16], and work of Khonzue et al. [3] relates to an optimum temperature range 50-60°C, showing that the temperature found in A. *japonicus* resembles other *Aspergillus* species.

The xylanase produced by *A. japonicus* remained stable at 45 and 50°C, retaining 64% activity with 1 hour of incubation at 45°C and had a half-life of 1 hour at 50°C (Figure 4). In studies with *A. terreus*, the xylanase was also thermotolerant at 45 and 50°C, but had half-life of only 36 minutes at 50°C [11]. And, *A. phoenicis* had a half-life of only 25 minutes at 50°C [15]. At 55°C, the xylanase of *A. japonicus* had a half-life of 15 minutes, while the xylanase of *A. carneus* M34 had only 7.5 minutes [25].

The addition of 5% glycerol or polyethyleneglycol somehow protected enzyme from thermal inactivation at 50°C (Figure 5). The xylanase activity increased 22% with glycerol, when compared to the control (without additives), with 1 hour of incubation. It was observed a half-life of 32 minutes to the control, and more than 60 minutes with glycerol (57% activity with 60 minutes). In studies of

Sandrim et al. [26], both xylanases were inactivated with a half-life (T_{half}) value of 2.3 min at 65°C, but the addition of 30% glycerol or polyethyleneglycol somewhat protected the enzyme from thermal inactivation.

Assays of cellulose biobleaching using xylanase

In recent years, a wide variety of studies about xylanases have been reported to have potential application in the bleaching process. Among the filamentous fungi used to produce xylanase, *Aspergillus* species are one of the most explored. For example, production of xylanase and cellulose pulp biobleaching processes have been reported for *Aspergillus niger* [3]; *A. terricola* Marchal and *A. ochraceus* [17]; *A. niger, A. niveus* and *A. ochraceus* [16]; *A. niveus* RP05 and *A. fumigatus* RP04 [5]; *A. nidulans* and *A. awamori* [27]; *A. fumigatus* [1], and *A. caespitosus* [26].

For estimation of the pulp bleaching potential of the crude xylanase from *A. japonicus* var *aculeatus*, two approaches were used: measurement of absorbance at 237 nm and estimation of kappa number. Cellulose pulp was pretreated with 10, 20 or 40 units of crude xylanase from *A. japonicus* per gram of dry cellulose pulp for 2 hours or with 10 units for different periods as 1, 2, 3 or 24 hours at 50°C, pH 5.2. Kappa number is an indication of the lignin content or bleach ability of wood pulp. Thus, it is expected to reduce the

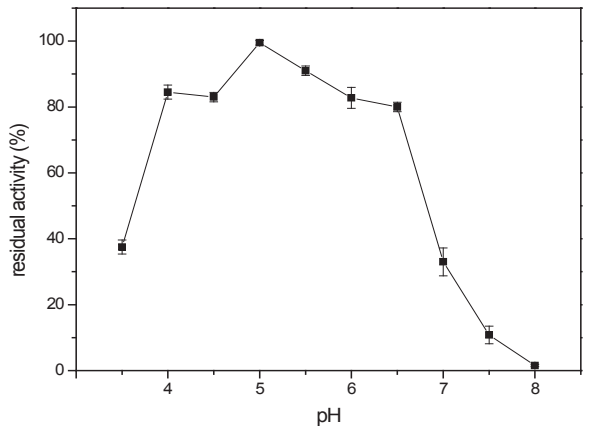

Figure 2: Xylanase pH was determined at 55°C using McIlvaine buffer on pH ranging from 3.5-8.0.

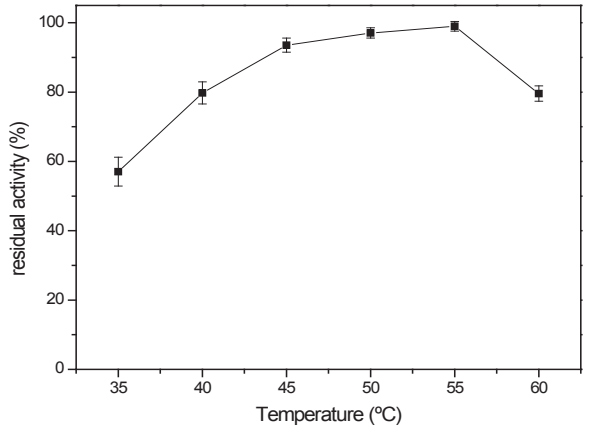

Figure 3: To test the effect of temperature, the xylanase activity was determined using McIlvaine buffer, pH 5.0, at temperatures ranging from 35-60°C.

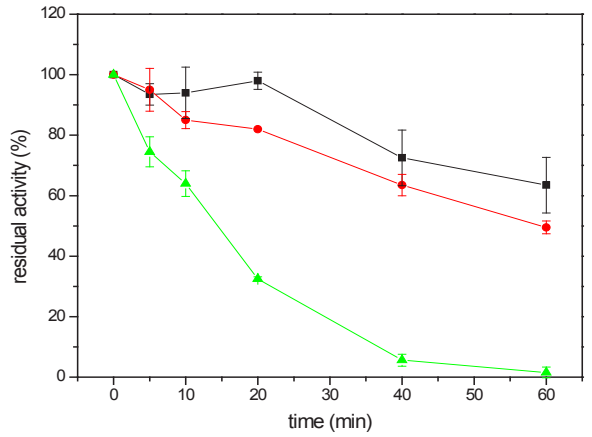

Figure 4: Thermostability of the xylanase of *A. japonicus* was determined using McIlvaine buffer pH 5.0 at 55°C after incubating the enzyme on temperatures of 45 (■), 50 (●) and 55°C (▲).

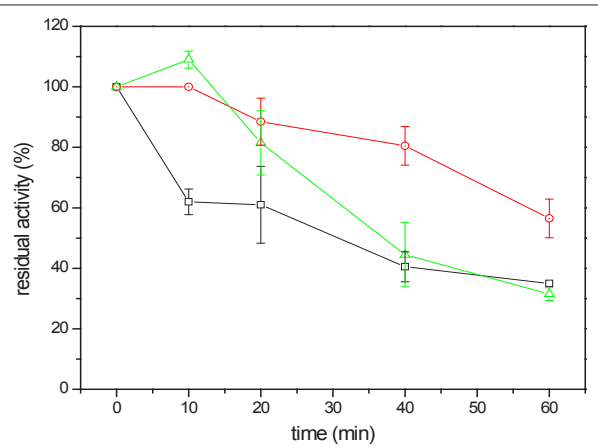

Figure 5: Thermostability of the xylanase at 50°C, without additives (□), and with 5% glycerol (○) and polyethyleneglycol (Δ).

kappa number after the enzyme treatment, and it is also expected the releasing of chromophores from lignin molecule (absorbing at 237nm). The filtrate extracted from the treated pulps were compared with the control pulp (not treated with xylanase), and it was observed that over the treatment time and with the increasing on enzyme concentration, there was a release of lignin (Table 3). The best result was obtained with 10 U/g dry pulp with 3 hours of treatment, which decreased 3.9 points in the kappa number comparing to the control, where the kappa efficiency corresponded to 25.2%. On the treatment of 2 hours, the xylanase (10 and 40 U/g dry pulp) reduced 2.1 points in the kappa number, which corresponded to a kappa efficiency of 18.8%. Using the xylanase, the brightness improved 2.2, 2.8 and 3.1 points in the treatment with 10, 20 and 40 U/g dry pulp, respectively, during 2 hours of treatment. The xylanase from *A. japonicus* was free of cellulase, not changing the viscosity of the pulp, meaning that the physical properties of cellulose were maintained. The delignification efficiency of *A. japonicus* in the treatment with 10 U/g dry pulp/ 1 hour (7.8%) was better than the described results with xylanases from *A. niveus* and *A. ochraceus* (6.5 and 7.5%, respectively) [16]. The xylanase of *A. caespitosus* (10 U/g dry pulp/2 hours) reduced kappa number only in 12.6% (xyl II) and 1.7% (xyl I) [26], while the

Table 3: Properties of pulp treated with xylanase produced by *A. japonicus*.

TREATMENT 1						
Xylanase (U/g dry pulp)	Time (h)	Treatment	Kappa number	Kappa efficiency (%)	Brightness (ISO)	A_{237}
10	2	Control	11.2	-	56.8	
		Treated	9.1	18.8	59.0	0.033
20	2	Control	11.2	-	56.8	
		Treated	9.4	16.1	59.6	0.066
40	2	Control	11.2	-	56.8	
		Treated	9.1	18.8	59.9	0.070
TREATMENT 2						
Xylanase (U/g dry pulp)	Time (h)	Treatment	Kappa number	Kappa efficiency (%)	Brightness (ISO)	A_{237}
10	1	Control	12.8	-	57.6	
		Treated	11.8	7.8	58.7	0.017
10	2	Control	11.2	-	56.1	
		Treated	9.1	18.8	58.3	0.027
10	3	Control	15.5	-	56.6	
		Treated	11.6	25.2	57.7	0.037
10	24	Control	12.2	-	57.0	
		Treated	10.6	13.1	57.2	0.040

The microorganism was grown on its standardized conditions. The controls corresponded to untreated samples. Cellulose pulp was pretreated with 10, 20 or 40 units of crude xylanase from *A. japonicus* per gram of dry cellulose pulp for 2 h, and with 10 units for 1, 2, 3 or 24 h at 50°C and pH 5.2.

A. japonicus xylanase kappa efficiency corresponded to 18.8%. Using the xylanase of *A. japonicus* (40 U/g dry pulp/ 2 hours), the brightness improved 3.1 points, while the xylanase of *A. ochraceus* (35 U/g dry pulp/ 2 hours) improved the brightness just 2.0 points [16].

Conclusions

The production of xylanase by *A. japonicus* using alternative carbon sources proved to be almost as efficient as when using the specific substrate, demonstrating that the use of alternative carbon sources shown viable, as well as favoring the cheaper of the cost of enzyme production, since the commercial xylan is extremely expensive for widespread use. The xylanase from *A. japonicus* showed to be relatively stable, demonstrating that it presents promising characteristics to be industrially applied on biobleaching of cellulose pulp process.

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Declaration of Interest

This work do not presents any conflict about financial interest, and all authors are in completely agreement with the submission. The agency that financered this project, and is properly quoted in acknowledgements.

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Anexos

(ARTIGO 2)

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Bioprocess and biotecnology: effect of xylanase from *Aspergillus niger* and *Aspergillus flavus* on pulp biobleaching and enzyme production using agroindustrial residues as substract

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Abstract

This study compares two xylanases produced by filamentous fungi such as *A. niger* and *A. flavus* using agroindustrial residues as substract and evaluated the effect of these enzymes on cellulose pulp biobleaching process. Wheat bran was the best carbon source for xylanase production by *A. niger* and *A. flavus*. The production of xylanase was 18 and 21% higher on wheat bran when we compare the xylanase production with xylan. At 50°C, the xylanase of *A. niger* retained over 85% activity with 2 h of incubation, and *A. flavus* had a half-life of more than 75 minutes. At 55°C, the xylanase produced by *A. niger* showed more stable than from *A. flavus* showing a half-life of more than 45 minutes. The xylanase activity of *A. niger* and *A. flavus* were somehow protected in the presence of glycerol 5% when compared to the control (without additives). On the biobleaching assay it was observed that the xylanase from *A. flavus* was more effective in comparison to *A. niger*. The kappa efficiency corresponded to 36.32 and 25.93, respectively. That is important to emphasize that the cellulase activity was either analyzed and significant levels were not detected, which explain why the viscosity was not significantly modified.

Keywords: *Aspergillus niger*; *Aspergillus flavus*; Agroindustrial residues; Wheat bran; Biobleaching

Introduction

Annually, large quantities of lignocellulosic wastes (agricultural and agroindustrial residues, like wheat bran, sugarcane bagasse, soybean, rice straw, corncob and orange peel) are generated through industrial processes such as breweries, paper-pulp, textile and timber industries. And their disposal is becoming a problem regarding space, causing environmental pollution because most of the wastes are disposed by burning. However, the plant biomass regarded as "wastes" are biodegradable and represent an inexpensive alternative source for microbial growth or enzymes production (Sepahy et al., 2011; Facchini et al., 2011; Okafor et al., 2007).

In the last decades, an increasing number of studies aimed to develop environmentally clean and non-toxic methods for industrial processes (Betini et al., 2009; Peixoto-Nogueira et al., 2009). The possibility of using a variety of agricultural residues is an attractive practice for the production of plant cell wall degrading hydrolytic enzymes, especially xylanase and cellulase, because this system could simulate the natural environment (Facchini et al., 2011, 2012).

The xylanase production has been reported for bacteria (Sepahy et al., 2011), actinomycetes (Garg et al., 2011) and fungi (Das et al., 2013; Sorgatto et al., 2012; Facchini et al., 2011, 2012). Filamentous fungi which produce xylanases are attracting greater attention than bacteria and yeast because they are particularly interesting from an industrial point of view, due to the fact they secrete much higher xylanolytic enzymes into the medium (Okafor et al., 2007; Polizeli et al., 2005).

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Among the filamentous fungi employed to produce xylanase, the *Aspergillus* genus is one of the most explored (Sandrim et al., 2005; Betini et al., 2009; Peixoto-Nogueira et al., 2009; Michelin et al., 2010). To date, however, there have been few reports of xylanases produced by *Aspergillus flavus*.

Xylanases produced by microorganisms has attracted a great deal of attention during the past few decades because of its potential biotechnological applications in various industries including food, feed, fuel, textile, and paper and pulp industries and in waste treatment (Yeasmin et al., 2011; Facchini et al., 2011; Michelin et al., 2010; Jiang et al., 2010).

An increasing awareness on environmental pollution has enforced the pulp and paper industries to strive for an alternate greener technology which will replace the use of harsh chemicals in their processes with microbial enzymes. The application of biocatalysts not only makes the process less toxic but also decreases costs associated with the production and consumption of resources (water, electricity, fuels) (Birijlall et al., 2011).

At present, bleaching process for kraft pulp uses large amounts of chlorine-based chemicals and sodium hydrosulfite, which are toxic, mutagenic, and persistent; they also cause numerous harmful disturbances in biological systems (Yeasmin et al., 2011). The enzymatic step in the process of cellulose pulp bleaching contributes to reduce the use of chlorine-containing reagents (Peixoto-Nogueira et al., 2009). Xylanases aid in catalyze the hydrolysis of glycosidic bonds in the xylan backbone, reducing the degree of polymerization of the substrate, enhancing the brightness of pulp (facilitating the chemical extraction of lignin from pulp in subsequent alkaline extraction) and diminish impurities. Cellulose-free, alkali and thermo-stable microbial xylanases are mostly ideal for biopulping and bleaching processes (Birijlall et al., 2011; Yeasmin et al., 2011; Peixoto-Nogueira et al., 2009).

This paper shows an important comparing of the xylanase production by *A. niger* and *A. flavus* using agroindustrial residues as carbon sources and tested their suitability for cellulose pulp biobleaching. These fungi produced xylanases with special characteristics, such as high pH stability and high optimum temperature, in comparison to others reported in the literature (Facchini et al., 2011; Sepahy et al., 2011). And the xylanase activity from *A. niger* was the most thermostable of three enzyme samples (*A. niger*, *A. flavus* and *A. japonicus* var *aculeatus*, studied before in our laboratory).

Materials and methods

Microorganisms

Aspergillus niger and *Aspergillus flavus* strains were isolated by us from soil samples, identified in the Federal University of Pernambuco – UFPE (PE, Brazil) and deposited in our laboratory fungi collection. Stock cultures were propagated at 30°C on slants of solid potato dextrose agar (PDA) media and stored at 4°C.

Xylanase production and enzyme extraction

Spores were inoculated into 125 ml Erlenmeyer flasks containing 25 ml media (Rizzatti et al., 2001) using 1% (w/v) of the desired carbon sources (xylan or agroindustrial residues such as wheat bran, rice bran, sugarcane bagasse or corncob). The cultures were incubated under orbital agitation (110 rpm) condition, at 30°C, during five (*A. niger*) or two days (*A. flavus*), already standardized in our laboratory. The media was subsequently vacuum-filtered using filter paper (Whatman nº 1) and the crude filtrate was used for the study of the extracellular enzyme.

Enzymatic assays and protein determination

The reaction mixture consisted in 500 μl of citrate-phosphate buffer (McIlvaine, 1921) pH 5.0 for both fungi containing 1% (w/v) of xylan (birchwood), and 500 μl of enzymatic extract appropriately diluted. Identical conditions of assay were employed for cellulase determination, using as substrate 1% (w/v) carboxymethylcellulose. The samples were incubated at 60°C to determine xylanase and cellulase activity. The amount of reducing sugar released was determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959), employing xylose (Sigma) (xylanase) and glucose (cellulase) as the standards. One unit of enzyme activity was defined as the amount of enzyme which releases 1 μmol of reducing sugar per minute under assay condition. Specific activities were expressed as U/mg of protein. Protein concentrations were determined by the Lowry et al. (1951), using bovine serum albumin (BSA) as the standard.

Effect of temperature and pH on enzyme activity

The effects of temperature and pH on xylanase activity were analyzed using crude extracts from *A. niger* and *A. flavus*. The optimum temperature and pH for *A. niger* and *A. flavus* were 60°C and 5.0, respectively (results not shown). The thermal stability was determined with enzymes incubated between 45 and 55°C for different periods (5 to 120 min). The influence of protectors on xylanolytic activities of *A. niger* and *A. flavus* was tested at 55 and 50°C, respectively, during 120 minutes in the presence of 5% glycerol or polyethyleneglycol. The pH stability was analyzed using McIlvaine buffer in the pH range 3.0 - 8.0 for 1 hour.

Biobleaching

The amount of enzyme used from *A. niger* or *A. flavus* for this treatment was 10 units of enzyme per gram of

dried cellulose pulp from *Eucalyptus grandis*. All calculations and procedures were determined according to the standard methods of Technical Association of the Pulp and Paper Industry (TAPPI test methods 1996). The consistency was determined on a percent dry weight basis. The volume of enzyme or distilled water was added until it reached a 10% pulp consistency. Crude xylanase extracts from *A. niger* and *A. flavus* were added to the treated pulp and the control was prepared by adding distilled water instead of enzyme. The samples were incubated inside sealed polyethylene bags at 55°C for 2 hours and after that, the treated cellulose pulps were filtered on a Büchner funnel, rinsed with 200 ml of distilled water and used for determination of kappa number and viscosity. The filtrate was used to analyze the liberation of aromatic compounds monitored by absorbance values at 237 and 465 nm.

Reproducibility of results

All results are expressed as the means of at least three independent experiments.

Results and discussion

Xylanase production using different agroindustrial residues and substrates

When different carbon sources were tested (Table 1) it was observed that the wheat bran 1% was the best carbon source for xylanase production by *A. niger* (12.76 U/mg of protein), followed by fine sugarcane bagasse 0.5% (10.64 U/mg of protein) and corncob 1% (10.00 U/mg of protein). The production of xylanase was 18 (oat spelt xylan 1%) and 21% (birchwood xylan 1%) higher on wheat bran 1% when we compare the xylanase production with xylan (10.43 and 10.10 U/mg of protein, respectively), which means that it can substitute the xylan efficiently. In studies with *A. fumigatus* RP04, the production of xylanase was only between 5 and 6% higher on agroindustrial residues (powdered corncob, wheat bran and crushed corncob) when compared with media containing birchwood xylan as carbon source (Peixoto-Nogueira et al., 2009). Although the xylanase production was elevated in birchwood xylan 0.5% (15.72 U/mg of protein), the utilization of an alternative carbon source, like wheat bran or sugarcane bagasse, still is viable due

Table 1 Effect of different carbon sources on xylanase production

Carbon sources	*Aspergillus niger*			*Aspergillus flavus*		
	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg of protein)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg of protein)
Glucose 1.0%	0.04	0.37	0.11 (±0.09)	0.18	0.79	0.23 (±0.03)
Rice bran 1.0%	2.77	0.67	4.13 (±0.45)	0.11	0.97	0.11 (±0.01)
Rice bran 0.5%	1.13	0.54	2.09 (±0.37)	0.09	0.97	0.09 (±0.01)
Rice straw 1.0%	0.97	0.45	2.16 (±0.10)	2.92	0.91	3.21 (±0.12)
Rice straw 0.5%	0.48	0.33	1.46 (±0.39)	1.87	0.71	2.63 (±0.02)
Coarse sugarcane bagasse 1.0%	5.49	0.72	7.63 (±0.45)	2.64	0.75	3.52 (±0.27)
Coarse sugarcane bagasse 0.5%	2.78	0.61	4.56 (±0.43)	0.63	0.66	0.96 (±0.14)
Fine sugarcane bagasse 1.0%	5.96	0.78	7.64 (±0.44)	5.53	0.72	7.68 (±0.29)
Fine sugarcane bagasse 0.5%	5.32	0.50	10.64 (±0.45)	2.33	0.97	2.40 (±0.09)
Corncob 1.0%	8.00	0.80	10.00 (±0.31)	3.22	0.86	3.74 (±0.07)
Corncob 0.5%	5.84	0.64	9.13 (±0.24)	1.65	0.62	2.67 (±0.44)
Wheat bran 1.0%	8.42	0.66	12.76 (±0.24)	11.57	1.44	8.03 (±0.48)
Wheat bran 0.5%	6.23	0.69	9.03 (±0.27)	10.26	1.18	8.70 (±0.03)
Xylan (oat spelt) 1.0%	6.05	0.58	10.43 (±0.31)	11.71	0.87	13.46 (±0.38)
Xylan (oat spelt) 0.5%	6.61	0.59	11.20 (±0.24)	10.38	0.87	11.94 (±0.41)
Xylan (birchwood) 1.0%	7.27	0.72	10.10 (±0.13)	11.22	0.55	20.40 (±0.21)
Xylan (birchwood) 0.5%	9.12	0.58	15.72 (±0.05)	11.64	0.74	15.72 (±0.13)
Corncob 0.5% + Fine sugarcane bagasse 0.5%	9.25	0.52	17.79 (±0.50)	3.59	0.76	4.72 (±0.02)
Wheat bran 0.5% + Fine sugarcane bagasse 0.5%	9.90	0.52	19.04 (±0.37)	10.77	1.23	8.75 (±0.37)
Wheat bran 0.5% + Corncob 0.5%	10.50	0.52	20.19 (±0.36)	11.92	1.07	11.14 (±0.01)

A. niger and *A. flavus* were grown on SR liquid media (Rizzatti et al., 2001) and different carbon sources, which were incubated for five and two days, respectively, at 30°C under orbital agitation (110 rpm) condition. The assays were performed at 60°C and McIlvaine buffer pH 5.0.

to its low cost for enzyme production, reducing these wastes in the environment. For *A. flavus*, the xylanase production was better induced by wheat bran 1 and 0.5% (8.03 and 8.70 U/mg of protein, respectively), followed by fine sugarcane bagasse 1% (7.68 U/mg of protein). The fine sugarcane bagasse was autoclaved, dried and ground. The other substrates tested did not presented significant results for both fungi.

The universal suitability of wheat bran as substrate is because of its cell-wall polysaccharides that contain 40% xylan, and it does not aggregate, even under high moisture conditions, providing a large surface area (Dhillon et al., 2011; Betini et al., 2009). It is why in literature can be found a lot of works of xylanase production using wheat bran, for several Aspergillus species like, *Aspergillus terreus* (Sorgatto et al., 2012); *Aspergillus niger* BCC14405 (Khonzue et al., 2011); *Aspergillus niger* BC-1 (Dhillon et al., 2011); *Aspergillus terricola* Marchal (Michelin et al., 2010); *Aspergillus foetidus* MTCC 4898 (Chapla et al., 2010); *Aspergillus niger* (Farinas et al., 2010); *Aspergillus niger* DFR-5 (Pal and Khanum, 2010); *Aspergillus niveus* RP05 (Peixoto-Nogueira et al., 2009); *Aspergillus niger*, *Aspergillus niveus* and *Aspergillus ochraceus* (Betini et al., 2009) and *Aspergillus ficuum* AF-98 (Fengxia et al., 2008). Xylanase production on corncob and sugarcane bagasse was also reported for *Aspergillus terreus* (Sorgatto et al., 2012), *Chaetomium* sp. CQ31 (Jiang et al., 2010), *Aspergillus foetidus* MTCC 4898 (Chapla et al., 2010), *Aspergillus terreus* (Lakshmi et al., 2009), *Thermomyces lanuginosus* MC 134 (Kumar et al., 2009) and *Aspergillus niger* ANL 301 (Okafor et al., 2007).

In the tests of influence of mixtures (1:1) of substrates, the xylanase production by *A. niger* was 22% higher on the mixture of wheat bran and corncob when compared to media containing xylan as carbon source. When we compare the xylanase production by *A. flavus* in this mixture, it was almost 45% lower than from *A. niger*. And comparing the xylanase production between the used fungi in the other mixtures, *A. flavus* presented a very lower production than *A. niger*.

In literature, different combinations of agroindustrial residues as carbon sources for xylanase production, has been reported for several fungi (Khonzue et al., 2011; Facchini et al., 2011; Dhillon et al., 2011; Pal and Khanum, 2010; Betini et al., 2009; Peixoto-Nogueira et al., 2009). *Aspergillus niger* DFR-5 had his highest xylanase production in media containing a mixture of wheat bran and soybean powder in a ratio 7:3 (Pal and Khanum, 2010), while *Aspergillus niger* BC-1 mixed with *T. reesei* Rut C-30 (ATCC 56765) had the highest activity with rice straw and wheat bran (3:2) (Dhillon et al., 2011). Facchini et al. (2011) found that a mixture of soybean meal and crushed corncob increased xylanolytic activity by only 8.5%. And in work using the fungus *A. niveus* and some agroindustrial residues, wheat bran mixed with corncob not improved the production of xylanase, but *A. niger* and *A. ochraceus* had an increase of approximately 20% (Betini et al., 2009).

For the enzyme characterization and the biobleaching study, there were separated the filtrates that more produced enzymes. And the cellulase activity of theses filtrates was determined and did not detect significant levels (data not shown).

Effect of temperature and pH on xylanase stability

The xylanases produced by *A. niger* and *A. flavus* remained totally stable at 45°C. At 50°C both xylanases were stable for the first twenty minutes, where the xylanase of *A. niger* (Figure 1A) still retained 85.72% activity with 2 hours of incubation, and *A. flavus* (Figure 1B) had a half-life of more than 75 minutes. At 55°C, the xylanase produced by *A. niger* showed more stable than from *A. flavus* showing a half-life of approximately 45 minutes. In studies with *A. casielus* (Kronbauer et al., 2007), *A. phoenicis* (Chipeta et al., 2005) and *A. giganteus* (Coelho and Carmona, 2003) the xylanases had half-life of only 50, 25 and 13 minutes at 50°C, respectively. The xylanase produced by *A. terreus* FSS129 had a residual activity of only 70% approximately with 2 hours of incubation at 50°C, and just retained 25.4% activity at 55°C after 1 hour exposure, while our *A. niger* still retained 38.2% activity (Bakri et al., 2010). And at 55°C the xylanase of *A. casielus* had a half-life of only 17 minutes (Kronbauer et al., 2007).

The influence of glycerol and polyethyleneglycol as enzyme protectors was also tested (Figure 1C,D). The addition of 5% glycerol somehow protected significantly the xylanase produced by *A. niger* from thermal inactivation at 55°C during all the incubation period. The xylanase activity increased 20.66% with 2 hours of incubation when compared to the control (without additives), and it was observed a half-life of approximately 32 minutes to the control and 60 minutes with glycerol (Figure 1C). And the xylanase activity of *A. flavus* increased 30% with 2 hours of incubation at 50°C in the presence of glycerol, which was not sufficient time to get the enzyme half-life (Figure 1D). The polyethyleneglycol was not effective as protector for both enzymes.

Regarding pH stability, it was verified that the xylanase from *A. niger* (Figure 1E) was totally stable at all the pHs tested, retaining more than 95% activity during 1 hour, while the xylanase from *A. flavus* (Figure 1F) had a small decrease of 10% at pH between 3.0 – 5.5, maintaining stable (100%) at higher pHs. The stability of the xylanases of *A. niger* and *A. flavus* under alkaline pH is very interesting when we remember that the biobleaching process with xylanases is done under alkaline conditions. Michelin et al. (2010) related a xylanase produced by *A. terricola* Marchal and *A. ochraceus* that retained more than 70% of

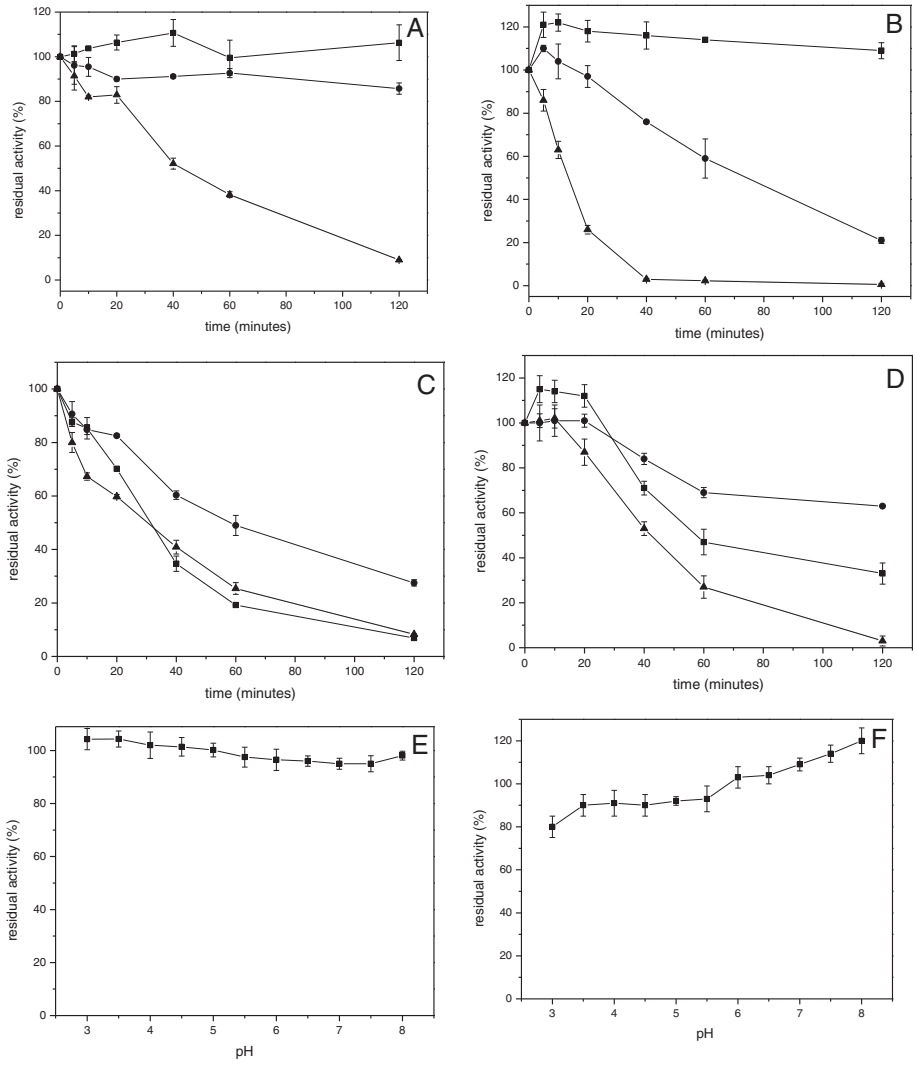

Figure 1 Characterization of the xylanases from *A. niger* and *A. flavus*. Thermostability of the xylanase of *A. niger* **(A)** and *A. flavus* **(B)** were determined using McIlvaine buffer pH 5.0 at 60°C after incubating the enzymes on temperatures of 45 (■), 50 (●) and 55°C (▲). Thermostability of the xylanases at 55 (*A. niger*) **(C)** and 50°C (*A. flavus*) **(D)**, without additives (■), and with 5% glycerol (●) and polyethyleneglycol (▲). pH stability of *A. niger* **(E)** and *A. flavus* **(F)** were determined incubating both enzymes at different pHs (3.0 - 8.0) at 0°C, during 1 hour and after the residual activities were determined at 60°C, pH 5.0.

its original activity in a pH range of 2.5 – 8.0 for 1 hour. The xylanase produced by *A. fumigatus* was stable just in pH from 6.0 to 8.0, while the xylanase from *A. niveus* was more stable from 4.5 to 6.0 (Peixoto-Nogueira et al., 2009). The xylanases of *A. niger*, *A. niveus* and *A. ochraceus* were stable in the range of pH 2 – 7 retaining more than 70 – 80% activity (Betini et al., 2009).

Assays of cellulose biobleaching using xylanase

To analyze the xylanases efficiency for cellulose pulp biobleaching assay, the cellulose pulp was clarified by *A. flavus* or *A. niger* crude extract. The procedures occurred

Table 2 Properties of pulp treated with xylanases produced by *A. niger* and *A. flavus*

Parameters	Control	*A. niger*	*A. flavus*
Kappa number	13.96	10.34	8.89
Kappa efficiency (%)	-	25.93	36.32
CST (%)	21.70	21.30	21.60
$A_{237\ nm}$	-	0.093	0.120
$A_{465\ nm}$	-	0.056	0.062

Biobleaching of bagasse pulp with 10 U xylanase g^{-1} of pulp in 0.5 M sodium citrate buffer (pH 6.5) at 55°C for 2 h.

at 55°C for 2 h and it was observed that the xylanase crude extract from *A. flavus* was more effective in comparison to *A. niger*. The kappa number reduced 5.07 and 3.62 points, respectively, which corresponds to 36.32 and 25.93 kappa efficiency, respectively (Table 2).

This efficiency can be also proved by cromophores liberation, which was redden at 237 and 465 nm and again was bigger when the cellulose pulp was clarified by *A. flavus* crude extract. That is important to emphasize that the cellulase activity was either analyzed and significant levels were not detected (data not shown), which explain why the viscosity was not significantly modified.

Comparing the used fungi that is clear that the xylanase from *A. flavus* is more effective in comparison to xylanase from *A. niger*. These advantages can also be seen when you compare the obtained results for *A. flavus* and *A. niger* to some data from the literature. The xylanase of *A. caespitosus* (10 U/g dry pulp/ 2 hours) reduced kappa number only in 12.6% (xyl II) and 1.7% (xyl I) (Sandrim et al., 2005), while the *A. flavus* and *A. niger* xylanases kappa efficiency corresponded to 36.32 and 25.93%, respectively. Medeiros et al. (2007) reported xylanases from *T. longibrachiatum*, *P. corylophilum* and *A. niger* that reduced only 1.1, 0.5 and 0.6 points the kappa number with 5 U/g dry pulp/ 4 hours; and *Aspergillus fumigatus* ABK9 reduced only 0.7, 1.2, 2.7, 3.3 and 4 points the kappa number, using 20, 40, 60, 80 and 100 U/g dry pulp/ 6 hours, respectively (Das et al., 2013). And Kumar et al. (2009) reported a xylanase (50 U/g dry pulp/ 3 hours) from *T. lanuginosus* MC 134 that reduced only 3.2 points the kappa number.

Conclusions

A. niger and *A. flavus* showed that are good xylanase producers and these enzymes can be obtained using alternative carbon sources such as wheat bran, corncob and fine sugarcane bagasse, that is important to emphasize that the xylanase production increased when a mix of residues was used, showing a better production than on media supplemented only by xylan, proving that they can substitute the specific substrate and consequently reduce the enzyme production cost, since the commercial xylan is extremely expensive for widespread use. The xylanase from A. *niger* showed to be stable for temperature and pH, while the *A. flavus* xylanase was not so stable for temperature but showed excellent stability at high pH (6–8), characteristics that are very important for the biobleaching of cellulose pulp process. On biobleaching assay the xylanases from *A. flavus* were more effective in comparison to xylanases from *A. niger*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NCAG: enzyme production and biobleaching processes, discussion of results, drafted the manuscript; MS: discussion of results; SCPN:biobleaching processes, drafted the manuscript, discussion of results; JHAB: biobleaching processes for pulp; FFZ: drafted the manuscript, discussion of results; MRM: drafted the manuscript; MLTMP: discussion of results, drafted the manuscript; GCG: discussion of results, drafted the manuscript. All authors read and approved the final manuscript.

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