COMPLEXO XILANOLÍTICO DE *Penicillium sclerotiorum*: PRODUÇÃO, PURIFICAÇÃO E CARACTERIZAÇÃO DE XILANASES E DE β-XILOSIDASES

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Profª. Drª. Marcia Regina Brochetto Braga

Aos meus pais Inácio e Regina,

Aos meus irmãos André Afonso, Andréia e Jean Carlo,

Dedico

"... e não há ninguém com espírito sem preconceitos que consiga estudar qualquer criatura viva, por mais humilde que seja, sem se deixar entusiasmar pela sua maravilhosa estrutura e propriedades."

Charles Darwin

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ABREVIATURAS

BSA – soro albumina bovina

CYA – meio Czapeck

CM – carboximetil

DEAE – dietilaminoetil

DNS – ácido 3,5-dinitrosalicílico

DTT – 1,4-ditiotreitol

EC – Comissão de Enzimas

EDTA – ácido etilenodiaminotetracético

GH – glicosil hidrolases

h – horas

IUBMB – União Internacional de Bioquímica e Biologia Molecular

kDa – kilo Dalton

K_i – constante de inibição

K_m – constante de Michaelis–Menten

MEA – ágar extrato de malte

min - minutos

MW – peso molecular

PAGE – eletroforese em gel de poliacrilamida

pI – ponto isoelétrico

PMSF – fluoreto de fenilmetilsulfônico

PNPX – p-nitrofenil–β-D–xilopiranosídeo

RAPD – polimorfismo de DNA amplificado ao acaso

rpm – rotações por minutos

SDS – dodecil sulfato de sódio

TLC – cromatografia em camada delgada

Tris – tris (hidroximetil) aminometano

$V_{máx}$ – velocidade máxima

RESUMO

Enzimas degradadoras de xilana, principal componente da hemicelulose, têm sido utilizadas em várias aplicações biotecnológicas, sendo que em alguns processos é necessário o uso de enzimas purificadas. Aplicações comerciais para as enzimas xilanolíticas envolvem a hidrólise enzimática da xilana, que está presente nos resíduos agrícolas e agroindustriais, sendo convertido a xilose e outros açúcares, que podem ser utilizados como substratos em processos fermentativos para a obtenção de proteínas celulares, combustíveis líquidos e outras substâncias químicas. A utilização destas enzimas também diminui a liberação de agentes poluentes em determinados efluentes, como da indústria de polpa de celulose. Xilanases e β-xilosidases são produzidas principalmente por bactérias e fungos, sendo que em geral, os fungos as produzem em níveis mais elevados. O gênero *Penicillium* apresenta espécies já caracterizadas como boas produtoras destas enzimas. Uma linhagem deste gênero, isolada de solo brasileiro, na região da Mata Atlântica e identificada como *Penicillium sclerotiorum* destacou-se por produzir xilanase em níveis elevados. O objetivo deste trabalho consistiu na avaliação da influência das condições de cultivo sobre a produção do complexo xilanolítico produzido por *P. sclerotiorum*, na caracterização físico-química desse sistema, bem como purificação e caracterização bioquímica de seus principais componentes. Por meio da determinação das condições ótimas de produção e da caracterização deste complexo enzimático foi possível estabelecer metodologias eficientes de purificação de xilanases e uma β-xilosidase. Através da caracterização físico-química das enzimas purificadas, foi possível avaliar seu potencial biotecnológico, visando futuras aplicações em processos industriais.

Palavras-chaves: caracterização enzimática; produção de enzimas; purificação de enzimas; *Penicillium sclerotiorum*; xilanases; β-xilosidases.

ABSTRACT

Xylan degrading enzymes, the main component of hemicellulose, have been used in various biotechnological applications, and in some cases the use of purified enzymes is necessary. Commercial applications of xylanolytic enzymes involve the enzymatic hydrolysis of xylan, which is present in agricultural and agro-industrial wastes, and can be converted to xylose and other sugars, which can be further used as substrates in fermentation processes to obtaining cellular protein, liquid fuels and other chemicals. The utilization of these enzymes also decreases the release of certain pollutants in wastewater, as in the pulp and paper industry. Xylanases and β-xilosidases are mainly produced by bacteria and fungi, and in general, the fungi produce them at higher levels. The genus *Penicillium* presents species already characterized as good producers of these enzymes. One strain of this genus isolated from Brazilian soil in the Mata Atlântica region and identified as *Penicillium sclerotiorum* attracted attention by producing xylanase in high levels. The objective of this study was to evaluate the influence of culture conditions on the production of the xylanolytic complex produced by *P. sclerotiorum* to characterize physical and chemical properties of this system as well to purify and biochemical characterize its main components. By determining optimal conditions for production and by characterizing this enzymatic complex it was possible to establish efficient methodologies for purification of xylanases and one β-xylosidase. Through their physical and chemical characterization, it was possible to evaluate their biotechnological potential for future applications in industrial processes.

Keywords: enzymatic characterization; enzymes production; enzymes purification; *Penicillium sclerotiorum*; xylanases; β-xylosidases.

SUMÁRIO

1. INTRODUÇÃO

Atualmente os processos biotecnológicos têm conquistado um lugar de destaque no desenvolvimento tecnológico mundial, exibindo características econômicas e operacionais que conferem vantagens em relação aos processos químicos convencionais. O emprego desses processos possibilita a produção de um grande número de metabólitos de interesse industrial, incluindo as enzimas, as quais ocupam um papel de destaque neste cenário. Enzimas comerciais são produzidas principalmente a partir de microrganismos, devido em grande parte à grande diversidade, facilidade de controle operacional e maior rendimento, quando comparado aos processos de obtenção de enzimas a partir de tecidos animais e vegetais. Ultimamente, o investimento em pesquisas que visam o desenvolvimento de novas aplicações de enzimas na indústria é considerável. As enzimas são aplicadas nos mais diversos setores, sobretudo nas indústrias de alimentos, papel, têxtil, ração animal e farmacêutica, com um mercado em crescente expansão.

A participação de enzimas capazes de degradar a xilana no mercado mundial está crescendo significativamente. Estas têm sido amplamente estudadas e são atualmente aplicadas nos mais diversos processos industriais. Dentre as principais, destacam-se as xilanases e β-xilosidases, as quais são empregadas para a produção de hidrolisados a partir de resíduos agroindustriais, no processamento de alimentos, bebidas e fibras vegetais, no enriquecimento nutricional de rações, no aumento na digestibilidade animal e no branqueamento do papel.

Sabe-se que a produção de enzimas é influenciada por um grande número de fatores, incluindo a taxa de crescimento do microrganismo em substratos apropriados, média de consumo de substratos, indução e repressão catabólica. Fatores físicos como pH e temperatura de cultivo também exercem grande efeito sobre a produção enzimática. Na natureza existe um grande número de microrganismos que eficientemente produzem enzimas xilanolíticas. Em virtude de seu imenso potencial vêm sendo realizadas pesquisas envolvendo enzimas xilanolíticas, produzidas a partir de microrganismos. Porém, para fins industriais, este processo de produção precisa ser otimizado, para obtenção de maiores níveis de atividade enzimática. O desenvolvimento de estudos para produção de xilanases e de β-xilosidases visando aumento de produtividade representa um alvo de investigação científica altamente relevante, tendo em vista a redução dos impactos ambientais e os benefícios econômicos gerados por sua aplicação em processos industriais.

Enzimas devem ser purificadas para o estudo de sua estrutura e propriedades. A determinação de suas características físico-químicas podem revelar importantes informações tanto sob o ponto de vista científico quanto biotecnológico. No entanto, a eficiência na purificação de proteínas depende da seleção de técnicas apropriadas. A escolha destas técnicas visa aperfeiçoar o desempenho, maximizar o rendimento e minimizar o número de passos requeridos para o isolamento da proteína de interesse. Portanto, a seleção das condições ótimas de produção, de técnicas apropriadas e eficientes para purificação e posterior caracterização de enzimas xilanolíticas fornecerá conhecimento essencial para o entendimento e aplicação desse sistema, ou dos microrganismos produtores dessas enzimas em processos industriais.

2. OBJETIVOS

2.1 Objetivo Geral

Otimizar a produção, purificar e caracterizar os principais componentes do sistema xilanolítico produzido por *P. sclerotiorum* visando futuras aplicações biotecnológicas.

2.2 Objetivos específicos

- Avaliar a influência da fonte de carbono, da agitação, do tempo de cultivo, do pH e da temperatura sobre a produção de xilanases e de β-xilosidases produzidas por *P. sclerotiorum*;
- Caracterizar estas atividades produzidas pelo referido fungo em condições otimizadas;
- Purificar os principais componentes desse complexo xilanolítico;
- Determinar as principais propriedades físico-químicas das enzimas xilanolíticas purificadas de *P. sclerotiorum*.

3. Revisão bibliográfica

3.1 Hemicelulases e xilana

A biomassa lignocelulósica é o maior constituinte das plantas, compreendendo a fonte orgânica mais abundante e renovável no mundo, com potencial para ser utilizada para a produção de alimentos e de energia. A maioria dos resíduos celulósicos da agricultura e florestas contém 20 a 50% de celulose e de 50 a 80% de hemicelulose e lignina. Estes são os principais constituintes da madeira, contudo outros constituintes poliméricos podem estar presentes em menores quantidades como amido e pectina (KULKARNI et al., 1999; BIELY, 2003b; SAHA, 2003a; ARO et al., 2005).

As paredes celulares dos vegetais diferem de acordo com as suas funções. A sua rigidez se deve a uma rede de microfibras que estão entrelaçadas por uma matrix de proteínas e de diferentes polissacarídeos tais como: celulose, pectina, hemiceluloses e outros (Figura 1) (UHLIG, 1998).

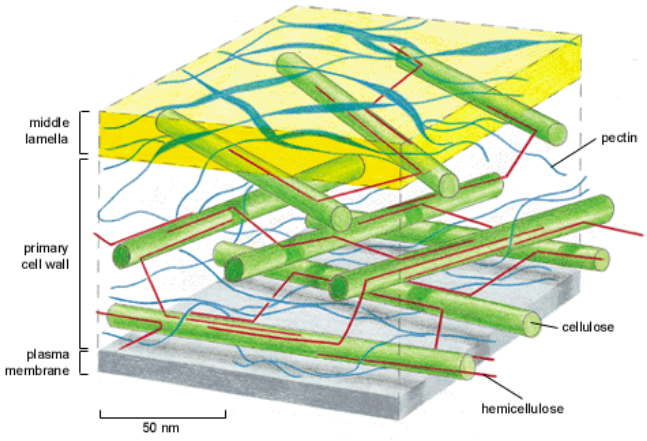

Figura 1 - Esquema da parede celular vegetal (McCann et al., 1990; http://quark.qmc.ufsc.br).

Em geral, a parede celular das plantas é subdividida em parede primária e secundária. A distribuição dos constituintes nessas camadas varia consideravelmente. A parede primária é uma camada delgada, permeável e flexível em tecidos fisiologicamente ativos (alburno), mas pode tornar-se altamente lignificada em células do cerne. As proporções relativas dos

componentes desta parede são: celuloses 30%, hemiceluloses 30%, pectinas 30% e proteínas 10%. Na parede celular primária, a estrutura microfibrilar da celulose é geralmente entrelaçada. Entre as paredes celulares de células vizinhas desta parede encontra-se a lamela média. A parede celular secundária é formada por uma seqüência de três lamelas, onde a camada central é normalmente mais espessa do que as outras. Esta é a camada externa da parede celular, depositada em alguns tipos de células sob a parede celular primária, após ter cessado o alongamento celular. Consiste basicamente de celulose (50-80%), hemicelulose (5-30%) e lignina (15-35%), onde as microfibrilas de celulose são depositadas de maneira altamente ordenada. A presença de lignina distingue a parede celular primária da secundária e confere rigidez a esta última, impedindo o crescimento celular. Como resultado, a maioria das características das fibras é derivada das propriedades e da integridade física dessa camada (FENGEL e WENEGER, 1989).

As hemiceluloses são genericamente definidas como heteropolissacarídeos não amiláceos e não celulósicos, que podem ser extraídos da parede celular dos vegetais superiores (FENGEL e WENEGER, 1989; SJÖSTRÖM, 1992). São polissacarídeos solúveis em reagentes alcalinos, de baixa massa molecular, apresentando entre 100 e 200 unidades glicosídicas. Representam cerca de 30 a 40 % dos carboidratos totais das células vegetais, correspondendo em torno de 40 % do peso seco da biomassa vegetal (COUGHAN et al., 1993; UHLIG, 1998; FERREIRA-FILHO, 2004). As hemiceluloses são estruturalmente mais parecidas com a celulose do que com a lignina e são depositadas na parede celular em um estágio anterior à lignificação (RAMOS, 2003). Diferentemente da celulose, a estrutura hemicelulósica não contém regiões cristalinas, sendo, portanto, mais susceptível à hidrólise química sob condições mais brandas. Entre a celulose, lignina e as hemiceluloses ocorrem interações covalentes e não covalentes, as quais são importantes para adesão e coesão das fibras, manutenção da integridade da parede celular vegetal e proteção da celulose contra o ataque de celulases (PRADE, 1995; UFFEN, 1997; BEG et al., 2001).

Nas plantas terrestres, as hemiceluloses podem ser compostas por uma variedade relativamente pequena de monossacarídeos, sendo os mais comuns: D-xilose, D-manose, D-glicose, L-arabinose, ácido 4-O-metil-D-glucurônico, ácido glucurônico e ácido galacturônico. São classificadas geralmente de acordo com os resíduos de açúcar presentes na molécula, como: D-galactana, D-xilana, L-arabinana e estão freqüentemente associadas aos polissacarídeos pécticos, porém esses últimos não estão incluídos no grupo das hemiceluloses (COLLINS et al., 2005). As ramificações e as cadeias laterais presentes na estrutura das

hemiceluloses interagem facilmente com a celulose, conferindo estabilidade e flexibilidade ao agregado (RAMOS, 2003). A variedade de ligações químicas e de ramificações, assim como presença de diferentes unidades monoméricas contribuem para a complexidade da estrutura hemicelulósica e suas distintas conformações (JACOBSEN e WYMAN, 2000).

As hemiceluloses presentes em folhosas e coníferas diferem significativamente entre si. As hemiceluloses de folhosas são compostas majoritariamente por heteroxilanas altamente acetiladas, geralmente classificadas como 4-O-glucuronoxilanas. Hexosanas também estão presentes, mas em quantidades muito pequenas como glucomananas. As coníferas, por sua vez, têm uma grande proporção de glucomananas acetiladas e galactoglucomanas, sendo que as xilanas correspondem apenas a uma pequena fração (RAMOS, 2003).

Dentre as hemiceluloses, a maior classe é a da xilana, que depois da celulose é o polissacarídeo mais abundante na madeira e nos resíduos agrícolas e agroindustriais. Na célula vegetal, a xilana está localizada na matriz da parede celular, formando uma interface entre a lignina e outros polissacarídeos, sendo facilmente encontrada na parede celular secundária (PRADE, 1995). Como os demais polissacarídeos de origem vegetal, a xilana apresenta grande variabilidade química e molecular, de espécie para espécie, o que está relacionado às suas funções em cada uma das plantas (EBRINGEROVÁ e HEINZE, 2000). Xilanas de diversas origens apresentam diferentes composições, tipos de ramificação e graus de polimerização, o que resulta em propriedades físicas e químicas distintas (PULS e SCHUSEI, 1993). De um modo geral, esse polissacarídeo tem uma estrutura linear que consiste de resíduos de xilose, unidos por ligações glicosídicas β-1,4 (Figura 2), podendo ainda apresentar resíduos substituintes como L-arabinofuranosil, acetil, glucuronosil e 4-O-metilglucuronosil (COUGHLAN e HAZLEWOOD, 1993; WONG e SADDLER, 1993). As cadeias laterais do polímero determinam à solubilidade, conformação e a reatividade da molécula de xilana e, portanto, influenciam grandemente o modo e extensão de sua clivagem enzimática. Assim, as ramificações desempenham papéis importantes na hidrólise da xilana, pois muitas vezes podem ocasionar impedimentos estéricos evitando a formação do complexo enzima-substrato (WONG et al., 1988).

A xilana é encontrada nas paredes celulares de todas as células vegetais de plantas terrestres e em quase todas as partes da planta (WONG et al., 1988), mas a distribuição dos vários componentes lignocelulósicos na parede celular das células vegetais depende do crescimento e diferenciação celular (WILKIE, 1979) e da espécie (SELVENDRAN, 1985).

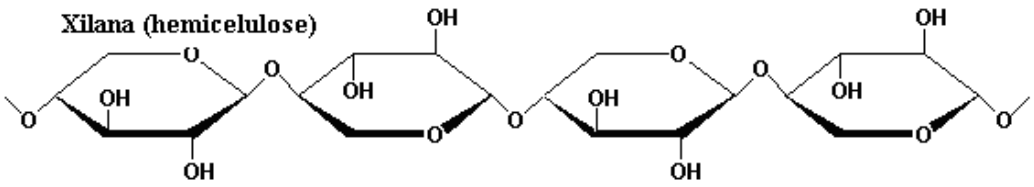

Figura 2 - Unidades de β-D-xilopiranose com ligações glicosídicas tipo β-1,4 (http://www.biosite.dk/leksikon/images/xylan.gif)

Este polissacarídeo é encontrado em grande quantidade não apenas na madeira dura (15 a 30% do conteúdo da parece celular) e madeira macia (7 a 10%), mas também em plantas anuais (acima de 30%), como o ramie, a cana-de-açúcar e o milho (TIMMEL, 1967; VIIKARI et al., 1994). Enquanto as hemiceluloses presentes nas madeiras duras são constituídas primariamente por xilanas, em madeiras macias as glucomananas são mais comuns (KUMAR et al., 2008). As xilanas estão presentes na forma parcialmente acetilada em várias plantas, sendo que existem ainda diferenças na acetilação da madeira macia e madeira dura. A xilana de madeira dura é altamente acetilada (70%), enquanto que a xilana de madeira macia não. A solubilidade parcial da xilana em água é devida principalmente à presença do grupo acetil (KULKARNI et al., 1999). A acetilação ocorre mais freqüentemente no O-3 do que no O-2, e, quanto maior o grau de acetilação, maior a solubilidade em água (BIELY, 1985).

A estrutura da xilana confere resistência à célula vegetal contra a degradação microbiana através das ligações entre as unidades de xilose, pela presença de grupos substituintes laterais e por meio de sua interação com outros polissacarídeos da parede celular. A existência de um complexo enzimático, tendo enzimas com funções especializadas, é uma estratégia que os microrganismos apresentam para alcançar a total hidrólise deste heteropolissacarídeo (KHENG e IBRAHIM, 2005).

3.2 A hidrólise da xilana e o sistema xilanolítico

Na natureza, a xilana é completamente hidrolisada a monossacarídeos pela ação conjunta de diferentes enzimas. A heterogeneidade da estrutura da xilana é responsável pela diversidade dessas enzimas. Este sistema enzimático, conhecido como sistema xilanolítico inclui principalmente β-1,4-D-xilanases (E.C. 3.2.18) e β-D-xilosidase (E.C 3.2.1.37), as quais atuam sobre a cadeia principal (SUNNA e ANTRANIKIAN, 1997; UFFEN, 1997; POLIZELI et al., 2005). Conjuntamente, xilanases e β-xilosidases hidrolizam xilooligômeros

a xilose que, em substituição a glicose, pode ser utilizada por microrganismos como fonte de carbono (FINELL et al., 2002).

As xilanases são as principais enzimas no processo de despolimerização da xilana, hidrolisando ligações glicosídicas internas ao longo de sua cadeia, liberando xilooligossacarídeos de vários tamanhos, o que resulta em um decréscimo do grau de polimerização do substrato (Figura 3). Geralmente, os produtos formados pela ação das endoxilanases são os xilooligossacarídeos maiores e, posteriormente, outros produtos podem ser liberados tais como: xilotriose e xilobiose (WONG et al., 1988; SUNNA e ANTRANIKIAN, 1997). O mecanismo de clivagem não é ao acaso e o ataque ao substrato depende do seu comprimento e grau de polimerização, bem como da presença de substituintes. Algumas xilanases atacam a cadeia principal com facilidade e mais rapidamente em regiões sem substituições, o que gera oligossacarídeos não substituídos e ramificados ou esterificados (BIELY, 1985; BIELY, 2003b), enquanto que outras enzimas dependem fortemente da presença de substituintes para exercerem sua ação (de VRIES e VISSER, 2001; BIELY, 2003b).

As β-D-xilosidases são exoglicosidases responsáveis pela hidrólise de xilooligossacarídeos menores e xilobiose, liberados pela ação das endoxilanases sobre a xilana, produzindo xilose (Figura 3). A especificidade dessas enzimas por pequenos oligossacarídeos de xilose é bastante elevada (SUNNA e ANTRANIKIAN, 1997). Estas enzimas desempenham um importante papel na degradação da xilana por remover os xilooligossacarídeos, os quais inibem a ação das endoxilanases e limitam, desta maneira, a hidrólise deste substrato (Sunna and Antranikian, 1997).

As endo-β-xilanases e β-xilosidases, apesar das suas especificidades e modos de ação diferentes, atuam com um alto grau de complementação na hidrólise da xilana (WONG et al., 1988) e o comprimento da cadeia e o grau de substituição são os fatores chaves que influenciam a taxa de hidrólise deste composto (LIAB et al., 2000). A capacidade hidrolítica das primeiras aumenta com o comprimento da cadeia, enquanto que a das segundas diminui com o comprimento da cadeia (PRADE, 1995). Grupos substituintes na cadeia principal da xilana podem limitar seu processo de hidrólise enzimática, sugerindo a existência de uma relação inversa entre o processo de hidrólise e o conteúdo destes resíduos (BRICE e MORRISON, 1982). Algumas xilanases purificadas são hábeis em hidrolisar xilotriose, mas não arabino-xilotriose, indicando que os substituintes α-L-arabinosil protegem as ligações xilosídicas das xilanases (TAKENISHI e TSUJISAKA, 1975). Todavia, é possível que os

resíduos substituintes tenham uma função positiva para que as ligações enzima-substrato ocorram, uma vez que muitas xilanases demonstram preferência em hidrolisar cadeias de xilana ramificadas (FREDERICK et al., 1985).

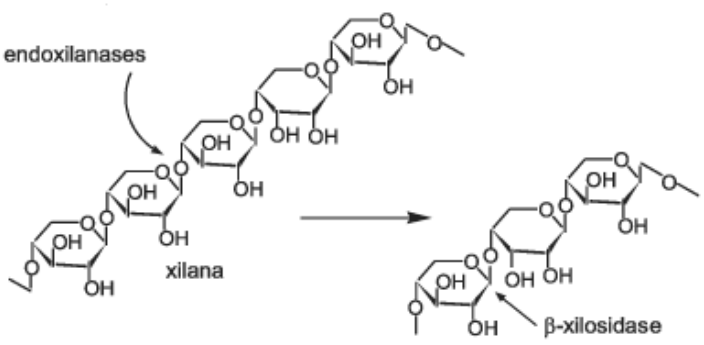

Figura 3 - Sítios de ação das xilanases e das β-xilosidases (Adaptado de GOMES et al., 2007).

Outras enzimas responsáveis por eliminar resíduos substituintes específicos também constituem o complexo xilanolítico, como α-arabinofuranosidases, acetilxilano esterases, α-D-glucuronidases, ácido ferúlico esterases e ácido p-cumárico esterases (FERREIRA-FILHO, 1994; COLLINS, 2005). Estas enzimas são conhecidas como enzimas auxiliares ou desramificantes e atuam juntamente com as xilanases e β-xilosidases no processo de despolimerização da xilana (Figura 4). Algumas atuam nas ligações entre um resíduo da cadeia principal e o grupo substituinte, enquanto outras quebram ligações entre os próprios grupos substituintes (de VRIES e VISSER, 2001). Vários estudos têm demonstrado a ação sinergística de enzimas fúngicas sobre xilanas com diferentes graus de polimerização (XIMENES et al., 1996; de VRIES et al., 2000; BIELY, 2003b; TUNCER e BALL, 2003). Sörensen et al. (2007) e Tuncer e Ball (2003) demonstraram que as xilanases, β-xilosidases e arabinofuranosidases são as enzimas chaves no processo de hidrólise da arabinoxilana.

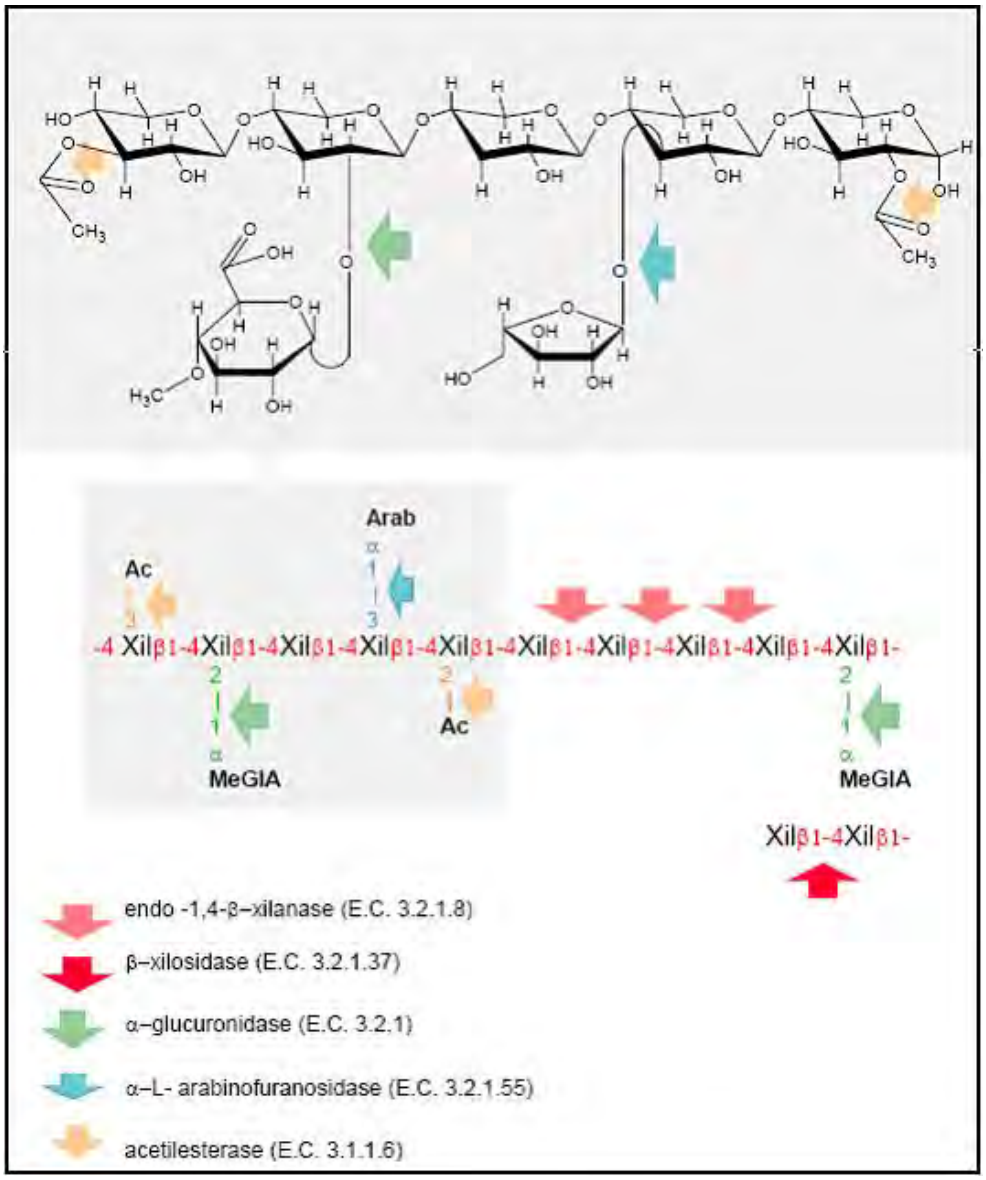

Figura 4 - Enzimas envolvidas no processo da degradação completa de uma arabinoxilana. Arab: arabinose; Ac: acetil; MeGlA: ácido 4-O-metilglucurônico; Xil: xilose (BIELY, 1985).

3.3 Classificação das xilanases e das β-xilosidases

Xilanases e β-xilosidades são glicosil hidrolases (GH) que constituem uma das maiores classes de enzimas com função na degradação de carboidratos (HENRISSAT e DAVIES, 2000). A classificação das glicosil hidrolases segue critérios baseados na especificidade pelo substrato, na similaridade de seqüência de aminoácidos e no modo de

ação (HENRISSAT e DAVIES, 1997). Quando a classificação deste grupo de enzimas é feita com base na especificidade pelo substrato, critérios estabelecidos pela União Internacional de Bioquímica e Biologia Molecular (IUBMB) devem ser seguidos. Conforme determina a IUBMB cada enzima recebe um código denominado "EC *number*" (*Enzyme Commission number*). Glicosil hidrolases são desta forma representadas através do código EC 3.2.1.x, onde x representa o substrato específico e, em alguns casos, também o mecanismo molecular ou o tipo de ligação entre as moléculas (HENRISSAT e DAVIES, 1997).

O sistema de classificação baseado na especificidade por substrato é um dos mais simples na categorização das glicosil hidrolases. Este sistema, no entanto, não é muito adequado às enzimas que agem sobre vários substratos, sendo isto particularmente relevante para aquelas glicosil hidrolases que atuam sobre polissacarídeos complexos presentes na natureza e que freqüentemente exibem ampla especificidade (DAVIES e HENRISSAT, 1995; HENRISSAT e DAVIES, 1997). Desta forma, a classificação de xilanases e β-xilosidases por este sistema é limitada, devido à heterogeneidade e complexidade da xilana (COLLINS et al., 2005). Além disso, este sistema não leva em conta as características estruturais das enzimas. Conseqüentemente, enzimas que agem sobre substratos similares podem ser incluídas numa mesma classe, sem que suas características estruturais, modo de ação e relações evolutivas sejam consideradas (DAVIES e HENRISSAT, 1995; HENRISSAT e DAVIES, 1997).

O critério de classificação de glicosil hidrolases atualmente empregado foi sugerido por Henrissat em 1991 (HENRISSAT e COUTINHO, 2001; COLLINS et al., 2005) e classifica as glicosil hidrolases dentro de famílias de acordo com a similaridade nas seqüências de aminoácidos. Glicosil hidrolases com domínios conservados são classificadas dentro de uma mesma família. Assim, quando as seqüências de aminoácidos de duas ou mais glicosil hidrolases são alinhadas em um domínio, estas são incluídas numa mesma categoria (HENRISSAT et al., 1995). Como a estrutura molecular e o mecanismo de ação de uma enzima estão relacionados à sua estrutura primária, esse sistema reflete características estruturais e mecanísticas (COLLINS et al., 2005).

Inicialmente esta classificação agrupou as glicosil hidrolases em 35 famílias, então designadas famílias 1 a 35 (HENRISSAT, 1991). No decorrer dos anos, houve um aumento significativo de seqüências de glicosil hidrolases depositadas nos bancos de dados (*EMBL/GenBank e SWISS-PROT*) e atualmente há o reconhecimento de 115 famílias. A descrição destas famílias está disponível no site http://www.cazy.org/ fam/acc_GH.html, o qual é atualizado regulamente.

Através desta classificação é possível agrupar enzimas com diferentes especificidades por substratos. Por outro lado, algumas enzimas que hidrolisam o mesmo substrato podem ser encontradas em diferentes famílias. No entanto, as estruturas tridimensionais das proteínas são mais conservadas do que suas seqüências de aminoácidos (DAVIES e HENRISSAT, 1995; HENRISSAT e DAVIES, 1997) e, por esta razão, a estrutura tridimensional das glicosil hidrolases vem sendo utilizada para agrupar as famílias em "clans". Atualmente existem 14 clans de famílias de glicosil hidrolases, designados por GH-A até GH-N, sendo o maior destes o clan GH-A, o qual agrupa 18 famílias relacionadas (HENRISSAT e DAVIES, 1997; COLLINS et al., 2005).

Neste novo sistema de classificação, as xilanases em sua maioria estão classificadas nas famílias 10 (F) e 11 (G). Entretanto, com menor freqüência, xilanases também podem ser encontradas nas famílias 5, 8 e 43, as quais diferem em suas propriedades físico-químicas, estruturas, modos de ação e especificidades por substratos (COLLINS et al., 2005; CHAVÉZ et al., 2006; CANTAREL et al., 2009). Xilanases de origem fúngica são encontradas apenas nas famílias 10 e 11 das glicosil hidrolases. Assim como xilanases, β-xilosidases são agrupadas em famílias de acordo com sua similaridade na seqüência de aminoácidos. Até o presente momento, β-xilosidases são encontradas nas famílias 3, 30, 39, 43, 51, 52 e 54, porém as de origem fúngica estão presentes somente nas famílias 3, 43 e 54 (CHAVÉZ et al., 2006; ENEYSKAYA et al., 2007; CANTAREL et al., 2009).

A família 10 apresenta xilanases de massas moleculares mais elevadas, a partir de 30-35 kDa, sendo que nesta família as xilanases são as enzimas predominantes. Embora demonstrem grande versatilidade, estas enzimas possuem baixa especificidade. São altamente ativas sobre xilooligossacarídeos curtos, indicando pequenos sítios de ligação ao substrato. Porém, estudos de especificidade por substratos têm revelado que estas xilanases também podem ser ativas sobre substratos celulósicos de baixa massa molecular, como aril-celobiosídeos e celo-oligossacarídeos. Estas xilanases exibem estrutura do tipo barril $(\beta/\alpha)_8$ e apresentam tipicamente baixo pI e atividade ótima em pH mais alcalinos (JEFFRIES, 1996; RYE e WITHERS, 2000; BIELY, 2003b; COLLINS et al., 2005). Xilanases desta família incluem enzimas produzidas por *Bacillus stearothermophilus*, *Cellvibrio japonicus*, *Penicillium simplicissimum*, *Pseudomonas fluorescens* e *Streptomyces lividans* (CANTAREL et al., 2009).

A família 11 é composta por xilanases com massas moleculares menores, ao redor de 20 kDa. Estas são altamente específicas, formando um grupo muito homogêneo de

enzimas, consistindo somente de xilanases ativas exclusivamente sobre substratos que contêm resíduos de D-xilose. Quando comparadas às xilanases da família 10, xilanases da família 11 são menos versáteis em relação à catálise, de forma que os produtos liberados por sua ação podem ser adicionalmente hidrolisados por xilanases da família 10. Em geral, as enzimas pertencentes à família 11 apresentam pH ótimo ao redor de 5, elevado pI e estrutura enovelada em rolo-β (JEFFRIES, 1996; RYE e WITHERS, 2000; BIELY, 2003b; COLLINS et al., 2005). Com relação ao mecanismo, pode-se observar uma similaridade entre as famílias 10 e 11, as quais contêm enzimas que apresentam dois resíduos de glutamato envolvidos na catálise, e atuam na hidrólise da ligação glicosídica com retenção da configuração anomérica por um mecanismo de dupla substituição. Xilanases produzidas por *Aspergillus niger*, *Bacillus circulans*, *Bacillus subtilis*, *Thermomyces lanuginosus*, *Trichoderma harzianum* e *Trichoderma reesei* estão incluídas na família 11 das glicosil hidrolases (CANTAREL et al., 2009).

A família 5 constitui o maior grupo, sendo formada por diversas hemicelulases e celulases, mas possuía apenas 8 xilanases em 2005 (COLLINS et al., 2005), totalizando atualmente 10 representantes, incluindo as produzidas por *Aeromonas punctata*, *Clostridium cellulovorans*, *Erwinia chripanthemi*, *Meloidogyne incognita* e *Prevotella ruminicola* (CANTAREL et al., 2009). Embora as características dessas enzimas ainda estejam sob investigação, podem ser verificadas grandes variações em suas propriedades catalíticas. Xilanases incluídas na família 5 apresentam o mesmo tipo de dobramento tridimensional que as xilanases da família 10, que corresponde ao barril $(\beta/\alpha)_8$, sendo portanto classificadas no mesmo clã, o GH-A. O mecanismo de ação das xilanases da família 5 é similar aos das xilanases das famílias 10 e 11 e da mesma forma que as últimas apresentam dois resíduos de glutamato envolvidos na catálise (RYE e WITHERS, 2000).

Xilanases classificadas na família 8 apresentam estrutura em forma de barril $(\alpha/\alpha)_6$, formado por seis α-hélices internas mais seis α-hélices externas, característica do clã GH-M. Essa família contém principalmente celulases, mas também quitosanases, liquenases e xilanases. Uma das xilanases dessa família apresenta como produtos da hidrólise da xilana xilotriose e xilotetrose e são mais ativas sobre cadeias longas de xilooligossacarídeos (COLLINS et al., 2005). Esta família inclui apenas xilanases de procariotos, como as produzidas por *Bacillus sp*., *Clostridium cellulolyticum*, *Clostridium thermocellum*, *Lactobacillus brevis*, *Pseudoalteromonas haloplanktis* e *Vibrio fischeri* (CANTAREL et al., 2009).

A família 43 das glicosil hidrolases é constituída por xilanases, β-xilosidases, arabinofuranosidases, arabinases e galactosidases. Em 2005 existia apenas uma xilanase classificada nesta família (COLLINS et al., 2005), mas atualmente a família 43 inclui um maior número de xilanases. O número de β-xilosidases nesta família é muito mais expressivo (CANTAREL et al., 2009). Enzimas da família 43 apresentam estruturas enoveladas do tipo hélice-β. Apenas xilanases de organismos procarióticos pertencem a essa família, incluindo as produzidas por *Caulobacter crescentus*, *Bacillus subtilis*, *Geobacillus stearothermophilus*, *Klebsiella pneumoniae*, *Lactococcus lactis*, *Paenibacillus polymyxa e Rhizobium leguminosarum*. Os eucariotos *Cochliobolus carbonum e Penicillium herquei* são alguns microrganismos produtores de β-xilosidases da família 43 (CANTAREL et al., 2009). Tanto as enzimas pertencentes à esta família quanto as da família 8 atuam tipicamente com inversão do centro anomérico e apresentam resíduos de glutamato e aspartato no sítio catalítico (RYE e WITHERS, 2000).

Com exceção da família 43, todas as outras famílias nas quais as β-xilosidases estão incluídas atuam pelo mecanismo de retenção da configuração anomérica do substrato (BIELY, 2003b). Glutamato e aspartato são os prováveis resíduos envolvidos na catálise pelas β-xilosidases pertencentes às famílias 3, 43, 52 e 54, enquanto que dois resíduos de glutamato podem estar envolvidos na catálise das β-xilosidases pertencentes às famílias 30, 39 e 51 (CANTAREL et al., 2009). A família 3 das glicosil hidrolases é constituída, além de β-xilosidases, por β-glicosidases, glucanases, arabinofuranosidases, entre outras. A maior proporção das β-xilosidases é classificada nesta família, a qual contém as produzidas por bactérias dos gêneros *Bacillus*, *Clostridium* e *Streptomyces*, além de β-xilosidases produzidas por plantas, como *Arabidopsis thaliana* e por fungos filamentosos dos gêneros *Aspergillus* e *Talaromyces*. As famílias 30 e 39 restringem-se a apenas β-xilosidases produzidas por bactérias, como as dos gêneros *Bifidobacterium*, *Caulobacter*, *Cellvibrio*, *Clostridium*, *Escherichia* e *Salmonella* na família 30, e principalmente por aquelas dos gêneros *Aeromonas*, *Bradyrhizobium*, *Burkholderia* e *Pseudomonas* na família 39, entre outros. Até o momento, a família 51 apresenta apenas uma β-xilosidase-arabinofuranosidase produzida por *Arabidopsis thaliana*, enquanto que a família 52 é constituída exclusivamente por 10 β-xilosidases derivadas de procariotos. Dentre as últimas, estão as produzidas por *Aeromonas puntacta*, *Geobacillus pallidus* e *Geobacillus stearothermophilus*. Porém, a família 54 constituída por arabinofuranosidases e β-xilosidases apresenta apenas uma β-xilosidase

fúngica, produzida por *Trichoderma koningii*. As estruturas das enzimas incluídas nesta família não estão relacionadas a nenhuma outra família, não constituindo desta forma nenhum clã (CANTAREL et al., 2009). Alguns aspectos sobre a clonagem gênica e a classificação das β-xilosidases fúngicas estão apresentados no capítulo 1 deste trabalho.

3.4 Fontes microbianas e propriedades das xilanases e β-xilosidases

Os microrganismos apresentam uma grande diversidade genética e desempenham funções únicas e cruciais na manutenção de ecossistemas, como componentes fundamentais de cadeias alimentares e dos ciclos biogeoquímicos (MEYERS, 1996). Como parte do ciclo do carbono, os principais constituintes da parede celular vegetal não se acumulam na natureza, uma vez que são degradados por sistemas enzimáticos especializados produzidos por microrganismos (BIELY, 1993; BIELY, 2003a). Desta maneira, as enzimas produzidas pela comunidade microbiana têm-se tornado objeto de intensa investigação no mundo inteiro (BEG et al., 2001). Foi provado que estas enzimas são muito eficientes em degradar materiais lignocelulósicos naturais, representando potenciais fontes de aplicações nos mais diversos setores industriais (HARUTA et al., 2002; KATO et al., 2004). Aliado a isso, enzimas microbianas são freqüentemente mais utilizadas que as derivadas de plantas ou animais por causa da grande variedade de atividades catalíticas disponíveis, da facilidade de manipulação genética, da obtenção de elevadas concentrações de enzimas através do ajuste das condições de cultivo, bem como por ser uma fonte regular isenta de flutuação sazonal e ao rápido crescimento dos microrganismos (TIMMIS e DEMAIN, 1998).

Devido à complexidade química das xilanas das plantas, não é surpreendente que os microrganismos produzam um arsenal de enzimas degradadoras deste polímero. Como essa coleção de enzimas ocorre e como elas interagem na célula ou nas populações celulares para degradar a xilana é pouco compreendido. Sistemas xilanolíticos completos são amplamente encontrados entre os microrganismos, sendo que produtores mais importantes destas enzimas geralmente pertencem aos gêneros *Aspergillus*, *Trichoderma*, *Streptomyces*, *Phanerochaete*, *Chytridiomyces*, *Fibrobacter*, *Clostridium*, *Pichia* e *Bacillus* (COLLINS et al., 2005). Estes utilizam essas enzimas como via alternativa para aumentar suas opções de fontes primárias de carbono, sendo que alguns deles também as utilizam como parte de um programa genético dirigido para infectar e colonizar células de plantas (PRADE, 1995).

Microrganismos produtores de enzimas do complexo xilanolítico ocorrem em diversos *habitats*, principalmente onde ocorrem acúmulo e deterioração da biomassa vegetal. Geralmente são saprofíticos, vivendo livremente no solo ou na água; alguns crescem anaerobicamente, outros aerobicamente. Alguns são mesofílicos, enquanto que outros vivem sob temperaturas extremas; muitos se associam simbioticamente ao trato digestório de ruminantes ou de insetos que se alimentam de madeira (UFFEN, 1997).

As xilanases são freqüentemente relatadas em fungos filamentosos e bactérias (COOLINS et al., 2005) e suas características físico-químicas variam amplamente. Porém, os fungos constituem a fonte mais comum de xilanases (DEKKER e RICHARDS, 1976), e seus sistemas enzimáticos têm sido os mais extensivamente estudados (BASTAWDE, 1992). Muitas bactérias e actinomicetos produzem xilanases quando se encontram em pH neutro ou próximo dele (SUBRAMANIYAN e PREMA, 2000). Na maioria das vezes, xilanases bacterianas apresentam menores níveis de atividade que as fúngicas e, geralmente, encontram-se retidas no espaço periplasmático (BIELY, 2003a; POLIZELI, 2005). Bactérias aeróbicas secretam um número moderado de enzimas xilanolíticas que produzem grande quantidade de oligossacarídeos no meio, e a quebra completa desses oligossacarídeos ocorre apenas intracelularmente. Essa estratégia impede que os produtos da degradação fiquem acessíveis aos microrganismos competidores (SHALLON e SHOHAM, 2003). Fungos fitopatogênicos produzem xilanases que degradam os componentes da parede celular do hospedeiro para penetrar nos tecidos vegetais (WONG et al., 1988; ANNIS e GOODWIN, 1997; KULKARNI et al., 1999; SUBRAMANIYAN e PREMA, 2002; SÁ-PEREIRA et al., 2003). Xilanases também são produzidas por fungos micorrízicos, atuando tanto na penetração das hifas nas células corticais, durante o estabelecimento da interface fungo-raiz, como na degradação de tecidos de plantas mortas no solo (BURKE e CAIRNEY, 1997; SÁ-PEREIRA et al., 2003).

Entre aquelas de origem fúngica, as massas moleculares variam de 11 a 100 kDa, e seu ponto isoelétrico de 3,5 a 8,9 (Tabela 1). O pH ótimo apresentado pelas xilanases fúngicas até agora descritas situa-se entre 4,5 e 7,0. A temperatura ótima para estas enzimas purificadas de fungos pode variar de 40 a 80 ºC, sendo que a maioria apresenta temperatura ótima de atividade a 50-55 ºC. A estabilidade térmica das xilanases é muito variável, estando diretamente relacionada com o microrganismo em estudo. A ocorrência de enzimas glicosiladas é um fenômeno comum observado na maior parte das xilanases fúngicas purificadas (KULKARNI et al., 1999).

A produção de β-xilosidases é relatada em fungos e bactérias, podendo ser extracelulares ou ligadas à membrana, dependendo do organismo e tempo de cultivo (WONG et al., 1988). Em várias espécies de bactérias e actinomicetos, elas estão exclusivamente associadas às células, enquanto que em outros, como *Aerobasidium pullulans*, β-xilosidases são encontradas tanto associadas às células, quanto extracelularmente. Porém, em relação às culturas fúngicas, estas enzimas normalmente permanecem associadas ao micélio nos estágios iniciais de crescimento, sendo posteriormente liberadas no meio por secreção ou através da lise celular (BIELY, 1985; POLIZELI et al., 2005). As de origem fúngica geralmente são enzimas com massa molecular e ponto isoelétrico variando de 60 a 360 kDa e 3,0 a 7,3, respectivamente (Tabela 2) (COUGHLAN e HAZLEWOOD, 1993). A temperatura ótima para as β-xilosidases purificadas varia de 30 a 80 ºC, sendo que a maioria apresenta temperatura ótima de atividade a 60 ºC. O pH ótimo de atividade para estas enzimas varia entre 2,5 e 6,5. β-Xilosidases com massas moleculares acima de 120 kDa geralmente correspondem a formas diméricas, triméricas ou tetraméricas (COUGHLAN et al., 1993).

A degradação de materiais hemicelulósicos pelos fungos ocorre necessariamente de forma extracelular, uma vez que os componentes desses materiais devem ser inicialmente despolimerizados até compostos menores, que são suscetíveis ao transporte pela membrana celular e ao metabolismo intracelular dos fungos envolvidos (FERRAZ et al., 2003). Via de regra, os fungos filamentosos sintetizam enzimas mais ativas em pH baixo quando comparadas às enzimas produzidas por bactérias. A utilização de fungos filamentosos é mais interessante sob o ponto de vista industrial, uma vez que suas enzimas são secretadas diretamente no meio em que se encontram, não necessitando de ruptura celular para sua liberação. Além disso, os extratos enzimáticos obtidos destas culturas geralmente possuem maior atividade do que aqueles provenientes do cultivo de bactérias. Adiconalmente, os fungos possuem a capacidade de produzir diferentes enzimas do complexo xilanolítico, o que permite hidrolisar não somente a cadeia principal da xilana, mas também suas ramificações (HALTRICH et al., 1996). Entretanto, enzimas xilanolíticas produzidas por fungos filamentosos geralmente estão associadas às celulases (STEINER et al., 1987), além de serem, freqüentemente, menos estáveis do que as xilanases e β-xilosidases de bactérias (KULKARNI et al., 1999).

Microrganismos normalmente produzem múltiplas xilanases. Este fenômeno não é considerado uma redundância, mas uma função especializada destes para obterem uma hidrólise mais efetiva do substrato (BEG et al., 2001).

Tabela 01 – Características bioquímicas de algumas xilanases fúngicas

ESPÉCIE	ISOFORMAS	Massa MOLECULAR (kDa)	GLICOSILA-ÇÃO (%)	pI	ATIVIDADE ÓTIMA		REFERÊNCIA
					pH	TEMPERATURA (°C)	
Acrophialophora nainiana		17	-	-	6,0	50	XIMENES et al., 1999
Aspergillus caespitosus	I	27	-	-	6,5-7,0	50-55	SANDRIM et al., 2005
	II	17,7			5,5-6,5	50-55	
Aspergillus fischeri		13	-	-	5,5	60	SENTHILKUMAR et al., 2004
Aspergillus foetidus		-	-	-	5,3	50	SHAH e MADAMWAR, 2005
Aspergillus giganteus	I	21	55,7	-	6,0-6,5	50	FIALHO e CARMONA, 2004
	II	24	61,6		6,0	50	
Aspergillus versicolor		19	71,0	5,4	6,0	55	CARMONA et al., 1998
Aspergillus versicolor		32	14,1	-	6,0-7,0	55	CARMONA et al., 2005
Claviceps purpurea	I	21,5	-	8,9	-	-	GIESBERT et al., 1998
	II	33,8		7,0			
Fusarium oxysporum		21,6		8,2	5,5	55	JORGE et al., 2005
Fusarium proliferatum		22,4	-	-	5,0-5,5	55	SAHA, 2002
Aspergillus ficuum		35,0	-		5,0	45	FENGXIA et al., 2008
Neurospora crassa	I	33,0	-	4,8	4,8	50	MISHRA et al., 1984
	II	30,0		4,5	4,8	50	
Paecilomyces themophila		25,8	21	-	7,0	75-80	LI et al., 2006

Penicillium canescens		31	-	-	5,9	55-61	SINITSYNA et al., 2003
Penicillium capsulatum		28,5		5,0	4,0	47-48	FERREIRA-FILHO et al., 1993
Penicillium chrysogenum		35	-	4,2	6,0	40	HASS et al., 1992
Penicillium citrinum		20	-	3,5	5,0	55	TANAKA et al., 2005
Penicillium citrinum		31,6	-	-	6,0	50	WAKIYAMA et al., 2008
Penicillium expansum		-	-	-	5,5	40	QUERIDO et al., 2006
Penicillium herquei		11	-	5,1	3,0	50	FUNAGUMA et al., 1991
Penicillium purpurogenum	A	33,0	-	8,6	7,0	60	BELANCIC et al., 1995
	B	23,0		5,9	3,5	50	
Penicillium sp. AHT1		21,0		-	6,0	50	SHOFIQUR-RAHMAN et al., 2003
Penicillium sp. 40		25	-	4,7	2,0	50	KIMURA et al., 2000
Penicillium wortmanni		100	-	5,0	3,3-4,0	-	DELEYN et al., 1978
Scytalidium thermophilum		16	-	-	5,0	60	ZANOELO et al., 2001
Trichoderma harzianum		18	-	-	5,0	45	SILVEIRA et al., 1999
Trichoderma inhamatum	I	19	-	-	5,0-55	50	SILVA, 2006
	II	21			5,5	45-55	

Tabela 02 – Características bioquímicas de algumas β-xilosidases fúngicas

ESPÉCIE	ISOFORMAS	MASSA MOLECULAR (kDa)	GLICOSILA-ÇÃO (%)	pI	ATIVIDADE ÓTIMA pH	ATIVIDADE ÓTIMA TEMPERATURA (°C)	REFERÊNCIA
Aspergillus. awamori K4		117	-	-	4,0	70	KURAKAKE et al., 1997
Aspergillus brasiliensis		-	-	-	5,0	75	PEDERSEN et al., 2007
Aspergillus carbonarius		100	-	4,4	4,0	60	KISS e KISS, 2000
Aspergillus fumigatus		-	-	-	5,4	70	LENARTOVICZ et al., 2003
Aspergillus japonicus		113,2	27,6	-	4,0	70	WAKIYAMA et al., 2008
Aspergillus japonicus		-	-	-	5,0	60	PEDERSEN et al., 2007
Aspergillus nidulans		180	-	-	5,0	50	KUMAR e RAMON, 1996
Aspergillus niger		78	-	-	6,5	42	JOHN et al., 1979
Aspergillus niger		-	-	-	5,0	75	PEDERSEN et al., 2007
Aspergillus. oryzae		-	-	-	4,0	60	KITAMOTO et al., 1999
Aspergillus phoenicis		132	43,5	3,7	4,0-4,5	75	RIZZATTI et al., 2001
Aspergillus pulverulentus	Xyl I	180	-	4,7	2,5-3,5	60	SULISTYO et al., 1995
	Xyl II	190		3,5	4,0-5,0	60	
Aspergillus sp.		-	-	-	6,5	80	CASTRO et al., 1997
Aspergillus versicolor	I	60	21	5,6	6,0	40	ANDRADE et al., 2004
	II	100	47	6,5	6,0	45	

Cochliobolus carbonum		42	-	-	5,5-6,5	37	RANSOM e WALTON, 1997
Fusarium proliferatum		91,2	-	-	4,5	60	SAHA et al., 2003b
Fusarium verticillioides		94,5	-	7,8	4,5	65	SAHA et al., 2001
Humicola grisea var. *thermoidea*		43	Não glicosilada	-	5,0	60	de ALMEIDA et al., 1995
Humicola grisea var. *thermoidea*		50	-	-	6,5	55	LEMBO et al., 2006
Neocallimastix frontalis		180	-	4,3	6,5	35	HEBRAUD e FEVRE, 1990
Paecilomyces thermophila		53,5	61,5	-	6,5	55	YAN et al., 2008
Penicillium herquei	S1 S2	103,7 37,5	-	-	4,0 6,5	50 30	ITO et al., 2003
Penicillium. sp. AHT-1		-	-	-	6,0	50	RAHMAN et al., 2003
Penicillium. wortmanni QM 7322		-	-	5,0	3,3-4,0	-	DELEYN e CLAEYSSENS, 1978
Penicillium. wortmanni IFO 7237	I II III IV	110 195 210 180	-	3,7 4,3 4,6 4,8	3,0-4,5	55-65	MATSUO et al., 1987
Scytalidium thermophilum		45	12	7,1	5,0	60	ZANOELO et al., 2004
Sporotrichum thermophile		45	-	4,2	7,0	50	KATAPODIS et al., 2006
Talaromyces emersonii		-	-	-	3,0-3,5	60	RASMUSSEM et al., 2006

Talaromyces thermophilus	97	-	-	7,0	50	GUERFALI et al., 2008
Termitomyces clypeatus	94	-	-	5,0	60	BHATTACHARYYA et al., 1997
Trichoderma harzianum	60	Pouco glicosilada		4,0-4,5	70	XIMENES et al., 1996
Trichoderma koningii G-39	104	-	4,6	3,5-4,0	55-60	LI et al., 2000
Trichoderma reesei	100	-	4,7	4,0	60	HERMANN et al., 1997
Trichoderma reesei	-	-	-	3,0-3,5	60	RASMUSSEM et al., 2006

A primeira função das múltiplas xilanases seria a de destruir a integridade das fibras de xilana, uma vez que a mesmo atua na coesão das fibras de celulose, e expor os outros componentes lignocelulósicos a outras hidrolases (WONG et al., 1988). É também possível que a significância de múltiplas xilanases esteja em sua habilidade de funcionar sob diferentes condições, visto que suas propriedades físico-químicas diferem substancialmente. A multiplicidade do sistema xilanolítico pode ser atribuída a diversos fatores, entre eles: processamento diferencial do mRNA, proteólise parcial e diferenças nos graus de amidação e glicosilação. A glicosilação geralmente é uma das modificações pós-traducionais mais relatadas. Em alguns fungos, a presença de xilanases múltiplas pode ser devida a produtos de diferentes alelos do mesmo gene, bem como à presença de aloenzimas (THOMSON, 1993; FERREIRA-FILHO, 1994; KULKARNI et al., 1999; POLIZELI et al., 2005). Porém, muitos casos de xilanases múltiplas parecem estar relacionados à multiplicidade genética (PRADE, 1995; UFFEN, 1997; POLIZELI et al., 2005). As três xilanases purificadas a partir do filtrado de cultura de *T. harzianum* tiveram suas composições em aminoácidos determinadas, sendo sugerido que estas devem ser produtos de genes distintos (WONG et al., 1986).

Esta mesma multiplicidade foi verificada em relação às β-xilosidases, onde diferentes enzimas apresentam funções especializadas (van PEIJ et al., 1998; ITO et al., 2003). Enzimas com propriedades distintas podem ser mais efetivas na hidrólise de xilobiose ou de xilooligossacarídeos substituídos por xilosil, ou oligossacarídeos constituídos por xilosil e outros resíduos. Estas diferentes isoformas podem também apresentar habilidades diferentes para interagir com as xilanases na hidrólise da xilana (WONG et al., 1988).

3.5 Regulação do sistema xilanolítico

Uma ampla literatura está disponível em relação à regulação de enzimas xilanolíticas fúngicas, especialmente para espécies de *Aspergillus* e *Trichoderma*. As enzimas xilanolíticas podem diferir em ocorrência e concentração dependendo das condições metabólicas da célula. Geralmente são enzimas indutivas, que podem ter sua síntese associada a mudanças nutricionais e favorecidas pelas condições de cultivo, pH e temperatura (PRADE, 1995; KULKARNI et al., 1999; BEG et al., 2001; SHALLOM AND SHOHAM, 2003).

Algumas xilanases são produzidas em menor quantidade, ou porque podem exercer funções que não são requeridas com freqüência para hidrólise de ligações que não ocorrem freqüentemente, ou porque as condições de cultivo não induzem sua produção, ou ainda porque são perdidas devido à degradação ou adsorção pelo substrato (WONG et al., 1988).

Em geral, a produção de xilanases e β-xilosidases é induzida quando os microrganismos são cultivados na presença de hemiceluloses, como xilana, arabinana, manana, ou na presença de compostos derivados destes polímeros (BAJPAI, 1997; BEG et al., 2001; ARO et al., 2005). Devido à sua elevada massa molecular, a xilana não pode entrar na célula e então não pode induzir diretamente a síntese das enzimas xilanolíticas. São os fragmentos da xilana (xilose, xilobiose e xilooligossacarídeos), que por terem baixo peso molecular, vão ter ação na indução de xilanases (BASTAWDE et al., 1992; PRADE, 1995; KULKARNI et al., 1999; BEG et al., 2001). Normalmente, este fenômeno é bastante complexo e o nível da resposta a um indutor individual varia com os organismos. Portanto, a expressão da xilanase em fungos está sujeita à indução pelo substrato (de VRIES e VISSER, 2001; ZEILINGER et al., 1996; XU et al., 2000; TONUKARI et al., 2002) e apenas poucos casos de expressão constitutiva de xilanases têm sido relatados (SRIVASTAVA e SRIVASTAVA, 1993; BEG et al., 2001; SHALLOM e SHOHAM, 2003).

A regulação da síntese de enzimas xilanolíticas também pode estar sujeita a repressão catabólica por glicose (BIELY, 1985; PRADE, 1995; de GROOT et al., 2003; PRATHUMPAI et al., 2004; ARO et al., 2005; POLIZELI et al., 2005). Outras fontes de carbono facilmente assimiláveis como a xilose também freqüentemente reprimem a produção de enzimas xilanolíticas. de Graaff et al. (1994) relataram que a repressão catabólica do gene da xilanase em *Aspergillus tubigensis* pareceu ser controlada em dois níveis, diretamente pela repressão do gene da transcrição e indiretamente, pela repressão do ativador de transcrição. O mesmo padrão de regulação foi observado em *A. niger* e *A. nidulans.*

Verifica-se que o nível de indução de enzimas xilanolíticas é bastante variável, de acordo com os diferentes microrganismos produtores (KULKARNI et al., 1999). Além disso, um indutor que produz a atividade máxima de xilanases em uma espécie pode, porém, ser inibidor da atividade em uma outra espécie (HRMOVÁ et al., 1991). Existem casos de microrganismos como *A. nidulans, Bacillus subtilis* e *Clostridium absonum* que produzem xilanases resistentes à repressão catabólica por xilose e glicose (PIÑAGA et al., 1994; SRIVASTAVA e SRIVASTAVA, 1993; RANI e NAND, 2000). de Vries et al. (1999) verificaram que a xilose em baixas concentrações induz a produção de enzimas xilanolíticas em *A. niger*, porém em concentrações mais elevadas reprime a síntese dessas enzimas. Desta maneira, é possível verificar que em diferentes concentrações determinadas substâncias podem atuar como indutores ou repressores catabólicos.

Segundo o mecanismo proposto para a indução de enzimas xilanolíticas (BIELY, 1985; HALTRICH et al. 1996; SUNNA e ANTRANIKIAN, 1997; KULKARNI et al., 1999;

POLIZELI et al., 2005), uma pequena quantidade de xilanase constitutiva é secretada no meio e degrada a xilana em xilooligossacarídeos e xilobiose que, após serem absorvidos pela célula, induzem os genes a produzirem xilanases extracelulares (Figura 5). As xilanases induzidas degradam então a xilana em xilooligossacarídeos e xilobiose. O acúmulo destes monômeros provenientes da ação da enzima, entretanto, faz com que atuem como repressores na produção da mesma. Os derivados dos substratos e produtos enzimáticos finais podem desempenhar, portanto, um papel positivo chave na indução da enzima, e também podem agir como inibidores dos produtos finais, possivelmente em concentrações mais elevadas.

Estudos realizados com linhagens de *Aspergillus* e *Trichoderma* indicam que a regulação dos genes codificadores de xilanases e celulases ocorrem a nível transcricional (van PEIJ et al., 1998). Dois principais elementos foram descritos na regulação da expressão por fontes de carbono: CreA, uma proteína regulatória, que atua na repressão catabólica (RUIJTER e VISSER, 1997; SHALLOM e SHOHAM, 2003) e XlnR, um ativador da transcrição (van PEIJ et al., 1998; SHALLOM e SHOHAM, 2003). Ruijter e Visser (1997) sugeriram que a proteína CREA liga-se especificamente a seqüências no promotor de muitos genes alvos, inibindo desta forma a transcrição. Em *Aspergillus* e *Trichoderma,* a funcionalidade de diversas regiões consenso para esses reguladores CreA e XlnR já foi demonstrada (van PEIJ et al., 1998; STRICKER et al., 2008).

Contrariamente a *Aspergillus* e *Trichoderma*, pouco é conhecido a respeito da regulação da expressão gênica em outros gêneros fúngicos, entre eles *Penicillium* (CHÁVEZ et al., 2006; DÍAZ et al., 2008). Diversas espécies de *Penicillium* produtoras de xilanases (*P. purpurogenum*, *P. canescens*, *P. chrysogenum*, *Penicillium funiculosum*) apresentam prováveis seqüências consenso para CreA e XlnR (CHAVÉS et al, 2006; DÍAZ et al., 2008), porém a funcionalidade desses possíveis fatores de transcrição foi até agora comprovada apenas em *P. purpurogenum* (DÍAZ et al., 2008).

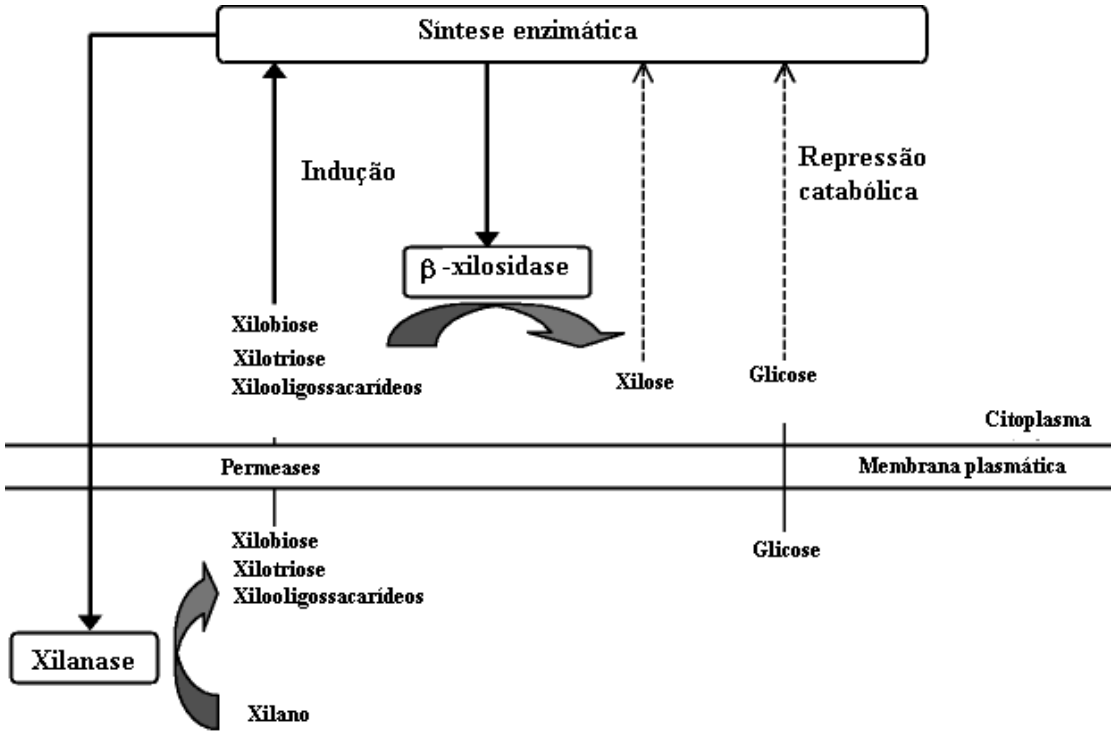

Figura 5 - Processo de regulação da biossíntese de enzimas xilanolíticas (POLIZELI et al., 2005).

3.6 Aplicações biotecnológicas das enzimas xilanolíticas

A seleção de microrganismos produtores de enzimas do complexo xilanolítico constitui uma etapa importante em processos biotecnológicos, nos quais estas enzimas podem ser empregadas. Enzimas que degradam a xilana são utilizadas em diversas aplicações biotecnológicas. Preparações comerciais de xilanases são obtidas em escala industrial, principalmente de fungos dos gêneros *Aspergillus* e *Trichoderma* (HALTRICH et al., 1996; POLIZELI et al., 2005).

Consideráveis quantidades de xilana resultantes das florestas são depositadas em córregos e rios, onde se tornam prejudiciais ao meio ambiente. Resíduos agrícolas ou agroindustriais constituídos em grande parte por xilana estão entre as maiores fontes de biomassa no mundo, que representam uma geração anual de 40 milhões de toneladas de resíduo lignocelulósico, gerando considerável prejuízo às atividades econômicas do setor agroindustrial e também ao meio ambiente (CANO e PALET, 2007). A conversão da xilana

em monossacarídeos pode ser realizada por dois processos: a hidrólise ácida e a enzimática; o primeiro, no entanto, libera resíduos tóxicos que podem impedir uma subseqüente fermentação microbiana. Buchholz et al. (1981) estudaram a hidrólise de materiais lignocelulósicos empregando ácidos ou bases e alcançaram bons resultados. No entanto, além destes autores, Ladisch (1979) constatou a formação de subprodutos indesejáveis, tais como furfural, hidroximetilfurfural, subprodutos da lignina e outros produtos inibidores da fermentação, bem como corrosão e problemas ambientais e econômicos. Além disso, a hidrólise ácida exige altos investimentos operacionais (LADISCH, 1979; TSAO, 1986). Desta maneira, a hidrólise enzimática oferece perspectivas para a degradação de resíduos hemicelulósicos por ser um processo mais específico, que pode ser conduzido sobre condições brandas, nas quais o produto final sempre é obtido sem a presença de subprodutos indesejáveis (REILLY, 1981; BIELY, 1985). Apesar dos baixos rendimentos (DAVID E FORNASIER, 1984), a hidrólise enzimática apresenta os benefícios adicionais de não gerar processos corrosivos, além dos açúcares permanecerem estáveis durante as diferentes condições do processo (HORTON et al., 1980).

Muito interesse tem sido dispensado a bioconversão de recursos renováveis em produtos economicamente importantes. A produção de etanol a partir de pentoses, como a xilose vem sendo amplamente estudada (SAHA, 2003a; KATAHIRA et al., 2004). Muitos microrganismos possuem a capacidade de converter xilose em etanol por fermentação, como *Pichia stipitis* e *Candida shehatae* (SCREENATH e JEFFRIES, 2000). Dentre os açúcares utilizados para a produção do etanol, a xilose representa apenas de 5 a 20%. Além disso, a xilose também vem sendo utilizada por microrganismos para a produção de xilitol, um poliálcool com poder adoçante semelhante ao da sacarose (PARAJÓ et al., 1998; SAHA, 2003a). Os resíduos lignocelulósicos podem ser ainda convertidos em outros aditivos alimentares, produtos químicos ou serem utilizados como fontes alternativas de energia (BAJPAI, 1999; HOWARD et al., 2003; SAHA, 2003a; FERREIRA FILHO, 2004; SÁNCHES, 2009).

Xilooligossacarídeos são oligômeros de açúcares formados por unidades de xilose, com diferentes graus de polimerização, normalmente apresentando de dois a cinco resíduos, que aparecem naturalmente em frutos, vegetais, leite e mel. Sua produção industrial é obtida através dos materiais lignocelulósicos, obtidos por meio da hidrólise ácida ou enzimática. Normalmente, os materiais típicos para a produção de xilooligossacarídeos são provenientes de uma base rica em xilana, com algumas cadeias heterocíclicas de éter, devendo ser

hidrolisada para gerar compostos degradados de cadeia longa (ENEYSKAYA et al., 2007). Para a produção de xilooligosacarídeos por enzimas, são necessários complexos enzimáticos contendo xilanases e/ou β-xilosidases. Estas enzimas podem ser diretamente adicionadas para a reação, imobilizadas ou produzidas *in situ* por microrganismos (BEG et al., 2001; LEMBO et al., 2001; XIONG et al., 2004; YOON et al., 2006). Através destes métodos, cadeias de xilooligosacarídeos podem ser produzidas, sendo preferíveis para uso em alimentos as cadeias que contenham entre 2 a 4 resíduos (van LOO et al., 1999).

Atualmente, xilooligosacarídeos são bastante utilizados como valiosos adoçantes ou aditivos na indústria de alimentos e também são empregados na indústria farmacêutica (YAMADA et al., 1993). Imaizumi et al. (1991) observaram que a suplementação de xilooligosacarídeos na dieta de ratos diabéticos foi capaz de reduzir a concentração de açúcares e de lipídeos no sangue destes animais. Em relação à saúde humana, xilooligosacarídeos são considerados pré-bióticos, uma vez que favorecem seletivamente o crescimento de pró-bióticos como *Lactobacillus* sp. e *Bifidobacterium bifidum*, promovendo uma série de benefícios, como a redução da constipação intestinal, o favorecimento da digestão e a absorção de nutrientes. Ainda, ajudam a prevenir infecções gastrointestinais, inibindo o crescimento de microrganismos patogênicos (GIBSON, 2004).

A aplicação de enzimas na ração animal tem aumentado consideravelmente nos últimos anos, devido ao custo cada vez maior das matérias-primas tradicionais e a busca por outros ingredientes alternativos (AHUJA et al., 2004). O uso de enzimas exógenas para reduzir os custos das rações representa, sem dúvida, uma das alternativas mais versáteis para auxiliar na melhoria de rentabilidade neste setor. A adição de enzimas em dietas de animais ruminantes e monogástricos para melhorar a eficiência da produção vem sendo estudada. Enzimas xilanolíticas são capazes de hidrolisar as hemiceluloses presentes, por exemplo, no trigo, milho e outros cereais, facilitando a digestão de todos os nutrientes e reduzindo a excreção de estrume, nitrogênio e fósforo. Os animais monogástricos não produzem enzimas capazes de degradar os polissacarídeos estruturais que compõem a parede celular das plantas, como a xilana e a celulose, tendo por isso um menor aproveitamento dos alimentos à base de cereais. Estas enzimas aumentam a energia metabolizável e diminuem a viscosidade dos alimentos, acarretando em ganho de peso desses animais (REIS et al., 2001; CONTE et al., 2002; TWOMEY et al., 2003; POLIZELI et al., 2005). Quanto mais energia o alimento possuir, mais ele será aproveitado podendo, com isso, reduzir seus níveis de inclusão nas dietas (CAMPESTRINI et al., 2005). Adicionalmente, a utilização de enzimas na ração

animal pode melhorar a eficiência da produção de carne e leite, bem como diminuir o impacto ambiental das explorações pecuária e agroindustrial, em virtude do aproveitamento de resíduos agroindustriais de baixo custo para produção de proteína animal (BHAT, 2000; AHUJA et al., 2004; GRAMINHA et al., 2008).

A indústria do papel e celulose vem utilizando enzimas do complexo xilanolítico em diversos processos (BIELY, 1985; VIIKARI et al., 1994; PRADE, 1995; SUNNA e ANTRANIKIAN, 1997; HAARHOFF et al., 1999; MEDEIROS et al., 2003; SUBRAMANIYAN e PREMA, 2002; POLIZELI et al., 2005). Nesse caso, o pré-tratamento com enzimas do complexo xilanolítico diminuiu o consumo de produtos químicos, principalmente cloro e óxido de cloro. O branqueamento enzimático resulta da clivagem das ligações entre lignina e carboidratos da estrutura da polpa (KULKARNI et al., 1999). Viikari et al. (1994) foi o primeiro a demonstrar que o tratamento da polpa de papel com hemicelulases pode substituir a necessidade de cloro para o seu branqueamento, sendo que outros pesquisadores também confirmaram esses resultados. Segundo Sunna e Antranikian (1997), estes processos de pré-branqueamento reduziram o uso de compostos clorados em até 30%. Como resultado, houve a diminuição de 15 a 20% na liberação de organoclorados altamente tóxicos para o meio ambiente, nos efluentes das indústrias de papel. Deste modo, xilanases e β-xilosidases vêm sendo utilizadas em pré-tratamentos como uma excelente alternativa para o branqueamento da polpa de papel (VIIKARI et al., 1994; OKSANEN et al., 2000; RAGHUKMAR et al., 2004). No entanto, deve-se ressaltar que, para maior eficiência desta aplicação específica das xilanases, deve-se trabalhar com extratos enzimáticos resistentes à alcalinidade e a alta temperatura, e totalmente livre de celulases (KULKARNI et al., 1999).

Enzimas xilanolíticas são bastante empregadas em pesquisas cientificas. Quando estas enzimas estão associadas às celulases, ambas tornam-se capazes de remover paredes celulares vegetais, liberando o seu conteúdo para ser utilizado em processos de produção de substâncias responsáveis por sabor ou aroma, na extração de polissacarídeos e proteínas de sementes e folhas, ou produzindo protoplastos de plantas superiores para uso em pesquisas genéticas. Além de serem o próprio objeto de estudo (WONG et al., 1988), enzimas xilanolíticas possuem papel importante na elucidação das estruturas complexas das xilanas (SAHA, 2003a). Ainda, oligossacarídeos produzidos a partir da atividade destas enzimas apresentam a potencialidade de aplicação direta como padrões para cromatografia (WONG et al., 1988; KULKARNI et al., 1999).

Na indústria de alimentos, a combinação de pectinases, celulases e hemicelulases, chamadas coletivamente de enzimas de maceração, é usada na extração e clarificação de sucos de frutas, apresentando vantagens sob os aspectos de rendimento, operacionalidade e qualidade do produto final (BIELY, 1985; BHAT, 2000; BEG et al., 2001; POLIZELI et al., 2005). Na panificação, as xilanases são aplicadas sobre a farinha de trigo, a qual é constituída por 2 a 3% (p/p) de arabinoxilanas. Estas absorvem cerca de 1/3 da água adicionada à massa, o que impede o desenvolvimento do glúten, reduz o volume do pão e prejudica a textura do mesmo. Assim, a aplicação de xilanases (arabinosidases) à farinha leva à liberação da água retida nas arabinoxilanas, melhorando o manuseio da massa e permitindo a obtenção de um produto final com maior volume e melhor estrutura de miolo, além de aumentar significativamente o tempo de prateleira das massas panificadas. Ainda, o emprego destes processos enzimáticos podem efetivamente substituir os aditivos usuais nas tecnologias empregadas na panificação como emulsificantes, oxidantes, malte de cevada ou de trigo. Na Europa, o uso tanto de brometo de potássio na massa do pão quanto de dióxido de enxofre na massa do biscoito foi vetado por reduzir a elasticidade do glúten. Em substituição a esses compostos estão as hemicelulases, incluindo as xilanases (KULKARNI et al., 1999; CAMACHO e AGUIAR, 2003). Na fabricação de cerveja ocorre liberação de longas cadeias arabinoxilanas, que aumentam a viscosidade, podendo deixar a cerveja turva. As xilanases auxiliam na solubilização das arabinoxilanas pela produção de oligossacarídeos menores, diminuindo sua viscosidade e conseqüentemente eliminando a turbidez da cerveja (van der BROECK et al., 1990; KULKARNI et al., 1999; DERVILLY et al., 2002).

Xilanases e β-xilosidases também são utilizadas, em aplicação conjunta com outras polissacaridases nas vinícolas, para reduzir a concentração de β-glicanas que aumentam à viscosidade dos mostos, prejudicando, consequentemente, a etapa da filtração que dificulta a clarificação dos vinhos (da SILVA, 1997). Benefícios adicionais obtidos através da aplicação destas enzimas na vinificação incluem a obtenção da cor dos vinhos e melhor estabilidade (BHAT, 2000).

Além disso, estas enzimas juntamente com celulases e/ou pectinases podem ser utilizadas na maceração de tecidos e no processamento de fibras vegetais, liquefação da mucilagem do café, extração de condimentos e pigmentos, óleos vegetais e amido (BIELY, 1985; WONG et al., 1988; PRADE, 1995; KULKARNI et al., 1999; COOLINS et al., 2005). A aplicação de enzimas na extração de óleos é uma alternativa interessante, por aumentar o rendimento do processo e eliminar o consumo de solventes, resultando em uma diminuição do consumo de energia e, consequentemente, no custo da extração (BARRIOS, 1990).

Métodos enzimáticos, envolvendo a utilização de xilanases, surgem como alternativas para o desenvolvimento de tecnologias mais viáveis, como a utilizada para o polimento do arroz (DAS et al., 2008). Além disso, enzimas xilanolíticas podem ser utilizadas nas indústrias de detergentes, de polissacarídeos farmacologicamente ativos, atuando como agentes antimicrobianos e na recuperação do óleo de minas terrestres (COLLINS et al., 2005).

3.7 O gênero *Penicillium* e a espécie *Penicillium sclerotiorum*

O gênero *Penicillium* foi descrito pela primeira vez por Link em 1809. As espécies incluídas neste gênero são ubíquas, de ampla distribuição por todo mundo. Muitas delas são geralmente saprofíticas na natureza e numerosas espécies são de particular valor para a humanidade. Muitos destes fungos habitam o solo, vários vivem sobre a matéria orgânica em decomposição, alguns ainda vivem em ambientes secos, madeiras e sementes, entre outros (MOSS, 1987; PITT, 2000).

Em muitas ocasiões, os alimentos destinados ao consumo humano são os *habitats* de *Penicillium* spp. Muitos são considerados patógenos de frutas e de algumas verduras frescas e cereais (BEUCHAT, 1987; PITT, 2000). Desta forma, algumas espécies de *Penicillium* possuem associações específicas com alimentos, o que facilita sua identificação, como as espécies pertencentes ao subgênero *Penicillium*, presentes nos cereais, ou como outras que apresentam maior grau de especialização: os patógenos de frutas *Penicillium digitatum* e *Penicillium italicum* nas frutas cítricas e *P. expansum* em peras, cerejas e outras frutas (FILTENBERG et al., 1996; PITT, 2000). Do ponto de vista econômico, as espécies deste gênero causam importantes perdas pela deterioração de cereais, frutas e outros alimentos durante o seu armazenamento. Muitas espécies do gênero são psicrófilas e podem deteriorar diferentes alimentos em baixas temperaturas, tornando-se um problema para os alimentos que são conservados sob refrigeração (BEUCHAT, 1987; PITT, 2000).

Um dos maiores efeitos deletérios causados por espécies de *Penicillium* para o homem e para os outros animais é a produção de micotoxinas, com diferentes efeitos tóxicos sobre os alimentos (WEBLEY et al., 1997). Algumas das mais importantes micotoxinas, como a citrinina, a ocratoxina e a patulina podem ser produzidas por espécies deste gênero (PITT et al., 1998; PITT, 2000).

Apenas uma única espécie de *Penicillium* é considerada patogênica para o homem e para os outros animais, denominada *Penicillium marneffei*. Este microrganismo é o agente

causal de uma micose endêmica na Ásia tropical, especialmente no norte da Tailândia, China, Hong Kong e Taiwan. Esta micose provoca um quadro de febre e perda de peso, acompanhados freqüentemente de erupções cutâneas em forma de pápulas. As infecções causadas por esta espécie geralmente comprometem pacientes imunodeprimidos (SUPPARATPINYO et al., 1994). Outras patologias que podem ser ocasionadas por fungos estão relacionadas à reações de hipersensibilidade. No caso de *Penicillium*, há relatos de pneumonia alérgica em trabalhadores de fábricas de queijo, expostos a uma grande carga de conídios de *Penicillium roqueforti* e *Penicillium verrucosum* (GUGLIELMINETTI et al., 2000).

A primeira e principal aplicação industrial envolvendo espécies do gênero *Penicillium* é a produção de penicilinas. O descobrimento deste metabólito produzido por *Penicillium notatum* (atualmente *P. chrysogenum*) foi realizado por Alexander Fleming, em 1928. Desde então, o estudo sobre o gênero aumentou consideravelmente, em busca de novas linhagens produtoras de elevada quantidade de penicilina e em busca de novos antibióticos. O uso da penicilina como agente terapêutico iniciou-se por volta de 1940 e, a partir de então, linhagens deste gênero são utilizadas nos processos industriais para a produção de antibióticos (PITT, 2000).

Algumas espécies de *Penicillium* são empregadas na produção de queijos. Dentre as espécies utilizadas para esta finalidade, incluem-se *P. roqueforti*, para a produção do queijo *Roquefort* e queijos azuis e *Penicillium camemberti*, para a produção do queijo *Camembert* e similares. Devido à produção de enzimas lipolíticas e proteolíticas, o crescimento do fungo confere ao queijo sua textura característica. O aroma e o sabor são conferidos pelos ácidos graxos livres, produtos da lipólise, bem como pelos produtos obtidos por meio da proteólise, além da formação de outros compostos aromáticos, entre os quais se destacam os álcoois e as cetonas (BEUCHAT, 1987). Algumas espécies de *Penicillium* são também empregadas na produção de derivados cárneos, especialmente em produtos fermentados e dessecados, como em salsichas e chouriço. Esta aplicação se reserva exclusivamente as espécies *Penicillium nalgiovence* e *P. chrysogenum* (GEISEN, 1993). Como no caso da produção de queijos, a atuação de enzimas lipolíticas e proteolíticas sobre os embutidos é capaz de melhorar suas propriedades organolépticas (GEISEN, 1993; GAREIS et al., 1999). Em países orientais, da África e da América do Sul, produtos fermentados são elaborados a partir de cereais que utilizam *Penicillium* spp. como fermentos naturais. O produto obtido geralmente é denominado *koji* e este pode ser utilizado como fermento na elaboração de outros produtos tradicionais (BEUCHAT, 1987).

As espécies do gênero *Penicillium*, assim como ocorre com outros gêneros de fungos microscópicos, compartilham muitas rotas metabólicas com animais e plantas, e possuem a capacidade de sintetizar metabólitos muito complexos (BRIGMANN e MADER, 1995). Muitos destes metabólitos são semelhantes a moléculas presentes em organismos superiores e alguns são utilizados como fármacos, como as benzodiazepinas (MANZONI e ROLLINI, 2002).

Outras espécies do gênero *Penicillium* são também utilizadas industrialmente para a produção de enzimas, principalmente pectinases, glicose oxidases e catalases (BEUCHAT, 1987; ADRIO e DEMAIN, 2003). A produção de enzimas xilanolíticas por *Penicillium* tem sido explorada em um grande número de espécies (CHÁVEZ et al., 2006). Segundo este mesmo autor, Penicillia constitui uma fonte rica de enzimas xilanolíticas. Particularmente notável foi o estudo conduzido por REESE et al. (1973), envolvendo 250 microrganismos de diversos gêneros para a análise da produção de β-xilosidases extracelulares. Destes, 11 foram selecionados, sendo que cinco deles pertenciam ao gênero *Penicillium*. Milagres et al. (1993) isolou uma linhagem de *Penicillium janthinellum* como excelente produtor de enzimas xilanolíticas. Abdel-sater e El-said (2001) selecionaram em placas de ágar contendo xilana um grande número de fungos filamentos isolados a partir de farelo de trigo e de arroz e bagaço de cana; dentre estes, cinco espécies pertenciam ao gênero *Penicillium*, os quais foram caracterizados como bons produtores de xilanases (*P. chrysogenum*, *Penicillium corylophilum*, *Penicillium duclauxii*, *P. funiculosum* e *Penicillium oxalicum*). Krogh et al. (2004), ao investigarem 12 espécies pertencentes ao gênero *Penicillium*, verificaram que todos os fungos analisados apresentam atividade xilanolítica, com destaque para a espécie *Penicillium persicinum*. Espécies de *Penicillium* produtoras de enzimas xilanolíticas foram isoladas dos mais diversos ambientes. Medeiros et al. (2003), ao isolarem fungos da Floresta Amazônica, detectaram três espécies de *Penicillium* produtoras de xilanases em níveis elevados. Bradner et al. (1999), após isolarem e identificarem fungos da Antártica encontraram níveis significantes de atividade xilanásica produzidos por *Penicillium hirsutum* e por *Penicillium commune*. Hou et al. (2006) isolaram uma linhagem de *P. chysogenum* a partir dos sedimentos do mar Amarelo, na China. Este fungo, bem adaptado a baixas temperaturas, apresentou elevadas atividades hidrolíticas, incluindo a atividade xilanase. Através destes e muitos outros estudos conduzidos pode-se concluir que o gênero *Penicillium* apresenta grande potencialidade em relação à produção de enzimas xilanolíticas.

A identificação das espécies de *Penicillium* apresenta bastante dificuldade devido à amplitude do gênero, a quantidade de espécies aparentemente semelhantes e, até mesmo, devido a grande variabilidade interespecífica (PITT, 2000; MAQUARDT e ABRAMSON, 2000; RUSSELL et al., 2004). Atualmente, espécies de *Penicillium* são identificadas principalmente por meio da análise de seus aspectos morfológicos e fisiológicos, em especial o diâmetro da colônia em meios específicos, e métodos moleculares não são muito utilizados. As informações taxonômicas obtidas através da análise de seqüências de DNA altamente conservadas, tais como as regiões ITS, podem com freqüência fornecer informações suficientes, embora não seja aconselhável utilizá-las como único critério para a caracterização. Nos casos em que as regiões conservadas apresentam pequenas variações, a identificação é confirmada utilizando-se marcadores moleculares como RAPD (Polimorfismo de DNA amplificado ao acaso) e pela análise do perfil de produção de metabólitos secundários (RUSSELL et al., 2004). Devido à inconsistência apresentada por muitas destas técnicas, quando empregadas isoladamente, recomenda-se em muitos casos uma combinação de métodos, a fim de se alcançar a correta identificação das estirpes (BOYSEN et al., 2000; RUSSELL et al., 2004). Freqüentemente, a análise da produção de metabólitos secundários complementa as informações morfológicas e genéticas (da SILVA et al., 1998).

Os dados obtidos a partir do seqüenciamento do DNA auxiliam na identificação, mas são mais adequados para estudos filogenéticos e menos satisfatórios em relação à identificação, quando comparados ao estudo dos aspectos morfológicos (FRISVAD e SAMSON, 2004). Desta forma, os métodos taxonômicos mais empregados e consistentes ainda são aqueles que recorrem às técnicas clássicas, baseadas na determinação das morfologias celulares em placas de Petri e na microscopia óptica comum (PITT, 2000; RUSSEL, 2004).

A estrutura que caracteriza as espécies do gênero *Penicillium* é o conidióforo que se apresenta sob a forma de um pincel (Figura 6). Da morfologia desta estrutura, deriva o nome do gênero (do latim *Penicillus,* "pincel pequeno"). Os conídios se apresentam em cadeias e são originados a partir de uma célula especializada: a fiálide. O conidióforo está unido ao micélio por meio da estipe. As células que dão suporte as fiálides denominam-se métulas e as que conferem suporte as métulas, quando presentes, denominam-se rama. Estas ramas surgem a partir da estipe, porém podem surgir a partir de outras ramas (PITT, 2000).

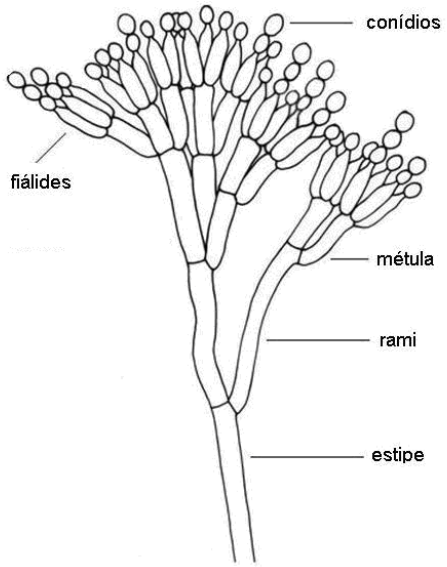

Figura 6 - Representação de um conidióforo hipotético encontrado em espécies de *Penicillium* (Adaptado de PITT, 2000).

O critério para a divisão entre os quatro subgêneros (*Aspergilloides*, *Furcatum*, *Biverticillium* e *Penicillium*) são os pontos de ramificação presentes entre a fiálide e a estipe até o eixo principal do conidióforo. Níveis de classificação abaixo de subgênero são determinados baseando-se na morfologia microscópica, na taxa de crescimento da colônia e na morfologia das colônias cultivadas em meios de cultura específicos (PITT, 2000).

P. sclerotiorum, pertencente ao subgênero *Aspergilloides*, foi primeiramente descrito por Beyma, em 1937. As espécies incluídas neste subgênero apresentam os conidióforos estritamente ou predominantemente monoverticilados, isto é, as fiálides estão unidas diretamente a estipe, apresentando apenas um único ponto de ramificação entre a própria estipe e a cadeia de conídios. Métulas são produzidas apenas por algumas espécies, mas estas são relativamente raras. *P. sclerotiorum* é um fungo que habita o solo, mas algumas vezes também ocorre em alimentos e em produtos têxteis, bem como em outras situações biodeteriorantes. Freqüentemente não germina abaixo de 5 ºC, porém, ocasionalmente, a germinação é observada nesta temperatura e seu crescimento não é detectado acima de 37 ºC. Colônias crescidas em meio Czapeck (CYA) ou em ágar extrato de malte (MEA) tipicamente medem de 30-40 mm de diâmetro, são moderadamente profundas, apresentando micélio esbranquiçado nas margens, tornando-se amarelo a alaranjado brilhante próximo ao centro (Figura 7a, b).

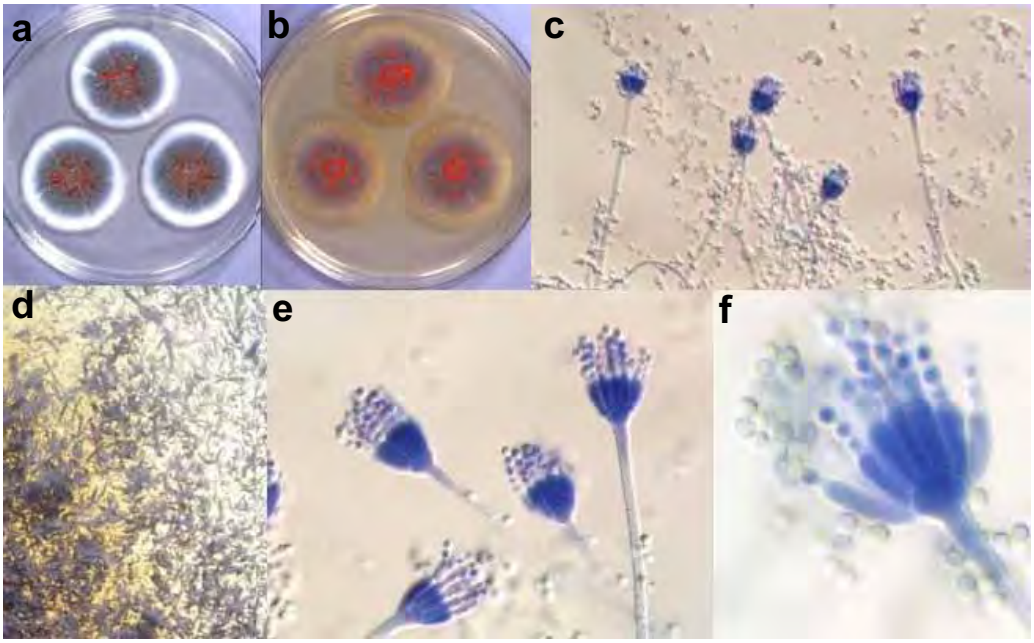

Figura 7 - *Penicillium sclerotiorum* van Beyma. Colônias crescidas em CYA (a) e em MEA (b) por 7 dias, a 25 ºC; conidióforos (c-f) (http://www.tamagawa.ac.jp).

A coloração alaranjada apresentada tanto pelo micélio aéreo quanto pelo micélio reverso é uma característica muito utilizada para distinguir *P. sclerotiorum* de outras espécies de *Penicillium* monoverticiladas. A conidiogênese é bastante esparsa, sendo os conídios geralmente verde-acizentados ou então turqueza-acizentados (Figura 7c-f). Pigmentos solúveis amarronzados ou amarelados são geralmente produzidos. Alguns isolados produzem estruturas de resistência denominadas escleródios, enquanto outros não (PITT et al., 2000). Este fungo tem sido alvo de investigação por sua capacidade de produzir alguns metabólitos biologicamente ativos, inclusive antibióticos (PAIRET et al., 1995; HARINANTENAINA et al., 2005). Dentre estes últimos, destaca-se a produção de esclerotiorina, a qual apresenta potente ação antimicrobiana contra *Micrococcus luteus*, *Bacillus cereus*, *Bacillus subtilis*, *Candida albicans*, *Klebsiella pneumonie*, *Eschericha coli*, *Salmonella typhimurium* e *Lysteria monocytogenes* (LUCAS et al., 2007).

Dentre 80 linhagens isoladas a partir do solo de região de Mata-Atlântica, na Estação Ecológica de Juréia-Itatins, Estado de São Paulo, Brasil, uma linhagem de *Penicillium sclerotiorum* apresentou elevados níveis de atividade xilanolítica. No entanto, nada se

conhecia sobre o sistema xilanolítico produzido por esta espécie, sendo seu estudo interessante a fim de se inferir possíveis aplicações biotecnológicas.

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5. CAPÍTULO 1:

β-xylosidases from filamentous fungi: an overview

KNOB, A.; TERRASAN, C.R.F.; CARMONA, E.C. **World Journal of Microbiology and Biotechnology** (enviado para publicação).

SUMMARY

β-xylosidases are hydrolytic enzymes which play an important role in xylan degradation, hydrolyzing xylobiose and xylooligosaccharides to xylose from the non-reducing end. Filamentous fungi are particularly interesting producers of this enzyme from an industrial point of view, due to the fact that they secrete β-xylosidases into the medium. Besides, fungal β-xylosidases are highly advantageous for their elevated activity levels and specificity. Interest in xylanolytic enzymes has been increasing, for their possible application in many biotechnological processes. This fact has driven the isolation, purification and characterization of several β-xylosidases. In this review, the mechanisms of action, substrate specificities, physicochemical characteristics, regulation at molecular level, molecular cloning and classification of filamentous fungal β-xylosidases are described. The potential industrial applications of fungal β-xylosidases will also be presented.

INTRODUCTION

Xylans are the most abundant renewable hemicelluloses of plant cell walls in hardwoods and cereals. Due to the heterogeneity and complex chemical nature of plant xylan, its complete breakdown requires the action of several hydrolytic enzymes with diverse specificities and modes of action. The most important xylanolytic enzymes are the endo-β-1,4-xylanase, which hydrolyses the insoluble xylan backbone into shorter, soluble xylo-oligosaccharides; and the β-xylosidase, which hydrolyses the soluble xylo-oligosaccharides and xylobiose from the nonreducing end liberating xylose. β-xylosidase plays an important role in xylan degradation by relieving the end product inhibition of endoxylanases. Furthermore, β-xylosidase is also effective in transglycosylation reactions wherein monosaccharide units or alcohols are attached to or cleaved from xylose units. In the last decade the interest in xylan-degrading enzymes has greatly increased due to their potential biotechnological applications. Owing to their importance, numerous research papers have been published in recent years, describing numerous β-xylosidases from new fungal sources. In the present review, the diversity of filamentous fungal β-xylosidases described to date is discussed.

XYLAN STRUCTURE AND β-XYLOSIDASE ACTIVITY

Xylan is the major constituent of hemicellulosic polysaccharide in cell walls of land plants, representing up to 30-35% of the total dry weight. It is the second most abundant natural resource and represents potentially renewable energy supply that could be utilized to improve the technology of plant biomass bioconversion into useful products (Aristodou and Penttilã, 2000; Beg *et al*., 2001).

This complex heteropolysaccharide consists of β-1,4-linked D-xylopyranosyl backbone and can be substituted with different side groups, such as L-arabinosyl, D-galactosyl, acetyl, feruloyl, p-coumaroyl and glucuronosyl residues. The frequency and composition of the branches are dependent on the xylan source (Beg *et al*., 2001; Biely, 2003; Saha, 2003a). The complete degradation of xylan is a key step in the carbon cycle and owing to its structural complexity, it requires the action of several hemicellulases (Beg *et al*., 2001; Shallom and Shoham, 2003; Collins *et al*., 2005). Complete xylan hydrolysis occurs by the synergistic action of enzymes, including endo-β-1,4-xylanase (EC 3.2.1.8), β-D-xylosidase

(EC 3.2.1.37), and several accessory enzymes, such as α-L-arabinofuranosidase (EC 3.2.1.55), β-glucuronidase (EC 3.2.1.139), acetylxylan esterase (EC 3.1.1.72), ferulic acid esterase (EC 3.1.1.73) and p-coumaric acid esterase (EC 3.1.1-) (Subramaniyan and Prema, 2002; Saha, 2003a; Polizeli *et al*., 2005).

According to Biely (1985; 1993) β-D-xylosidases can be recognized and classified as xylobiases and exo-1,4-β-xylanases in function of their relative affinities for xylobiose and larger xylooligosaccharides, respectively. Recent works suggest, however, that β-xylosidases can be defined as enzymes that catalyze the cleavage of xylobiose and attack the non reducing ends of short xylooligosaccharides to liberate xylose (Sorensen *et al*., 2003; Rasmussem *et al*., 2006). Due to its catalytic action, β-xylosidase plays an important role in xylan degradation by removing the end product that inhibits the endoxylanases and limits the xylan hydrolysis (Sunna and Antranikian, 1997).

Microorganisms are primarily responsible for xylan degradation in nature. Thus, β-xylosidases have been described from a variety of them, including fungi and bacteria. Filamentous fungi are widely used as enzyme producers and are generally considered more potent xylanolytic producers than bacteria and yeast (Haltrich *et al.*, 1996; Polizeli *et al*., 2005). Many of the β-xylosidases described showed mode of action that did not corresponded to the industrial needs. In particular, β-xylosidases with high specificity for xylooligosaccharides and low specificities for other substrates are desirable. Despite, fungal β-xylosidases are highly advantageous for their activity levels and specificity.

β-XYLOSIDASE LOCALIZATION

Contrarily to xylanases, which are always secreted by cells into the surrounding medium, β-xylosidases may be extracellular or cell-bound depending on the microorganism and the culture conditions (Lenartovicz *et al*., 2003). For example, in almost all bacteria and yeast, β-xylosidase is cell-associated and is considered to occur in the cytosol in a soluble form (Bajpai, 1997). In these microorganisms, xylooligosaccharides must enter the cell before hydrolysis (Biely, 1985). Conversely, filamentous fungi β-xylosidases remain associated with the mycelia during early stages of growth and can be released later into the medium, either by true secretion or as a result of cell lysis (Wong and Saddler, 1992). This way, several fungal β-xylosidases have been recovered in the extracellular medium (Bhattacharyya *et al*., 1997; Kiss and Kiss, 2000; Rizzatti *et al*., 2001; Saha, 2001; Lenartovicz *et al*., 2003; Yan *et al*.,

2008), while other fungal β-xylosidases remain cell associated during all growth period (Kumar and Ramón, 1996; Ito *et al*., 2003; Lembo *et al*., 2006).

TRANSXYLOSYLATION CAPACITY

Transxylosylation activity is the ability to form new glycosidic bonds yielding longer oligosaccharide chains (transglycosylation). According to Pan *et al*. (2001) one characteristic of fungal β-xylosidase is its transxylosylation activity, which makes it widely employed in biotechnology. The transfer reactions can be resulted of the complex structure of xylans. The hydrolytic activity would be restrained by xylan side chains, whereas the presence of high amounts of small sugars in the reaction mixture would encourage the transglycosylation activity (Kambourova et al., 2007).

Glycosides such as alkyl-β-xyloside, which have attracted special interest because of their physiological and physical usefulness, can be prepared and synthesized by β-xylosidase transfer reaction (Sulistyo *et al*., 1995). This transglycosylating activity has also been employed as an effective tool in the enzymatic synthesis of β-xylanase substrates (Eneyskaya *et al*., 2003) and oligosaccharides modified with chromophoric or fluorogenic groups (Mala *et al*., 1999; Zeng *et al*., 2000), due to its high selectivity and stereospecificity.

The formation of β-1,3 or β-1,4 xylobiose by the transfer reaction of a fungal β-xylosidase purified from commercial hemicellulase with phenyl-β-D-xyloside as substrate was first reported by Claeyssens *et al*. (1966). Further studies reported a great number of fungal β-xylosidases with transxylosylating ability (Shinoyama *et al*., 1988; Iizuka *et al*., 1992; Sulistyo *et al*., 1995; Herrmann *et al*. ,1997; Matsuo *et al*., 1998; Ito *et al*., 2003; Eneyskaya *et al*., 2007). Transglycosylation activity was also reported in filamentous fungal xylanases (Christakopoulos *et al*., 1996; Jiang *et al*., 2004).

Many β-xylosidases catalyze the transfer of xylopyranosyl residues to acceptors other than water, such as glycerol or other monosaccharides (Herrmann *et al*., 1997). The β-xylosidase S1 purified from *Penicillium herquei* presented transxylosylation activity, using xylobiose as donor and alcohols (methanol, ethanol, isopropanol, butanol and glycerol) as acceptors (Ito *et al*., 2003). Studies on transxylosylation demonstrated that the β-xylosidase from *Aspergillus niger* is effective for the preparation of transfer products from xylooligosaccharides to alcohols because of certain advantageous properties, such as its high

stability in organic solvents, strong transfer activity and simplicity of operation (Shinoyama *et al*., 1988). Sulistyo *et al*. (1995) reported that β-xylosidases from *Aspergillus pulverulentus* have broad acceptor specificity in transferring the xylosyl residue of xylooligosaccharides to various acceptors, such as alcohols and phenolic compounds. The β-xylosidase from *Aspergillus awamori* X-100 revealed a remarkable transglycosylating ability to produce xylooligosaccharides with degree of polymerization higher than 7 (Eneyskaya *et al*., 2007)

MODE OF ACTION AND SPECIFICITY

Hemicellulases are mainly glycoside hydrolases (EC 3.2.1–3.2.3), a widespread group of enzymes that hydrolyze glycosidic bonds between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety (Davies and Henrissat, 1995). The glycosidic bond is one of the most stable bonds in nature, with a half-life of over 5 million years (Wolfenden *et al*., 1988). Glycoside hydrolases can accelerate the hydrolysis of the glycosidic bond by more than 10^{17}-fold, making them the most efficient catalysts known (Davies *et al*., 1998).

β-xylosidases, as all glycosidases, hydrolyze the glycosidic bonds by one of two major mechanisms, giving rise to either an overall retention or an overall inversion of the configuration of the anomeric substrate carbon (Sinnott, 1990). In both mechanisms, the hydrolysis usually requires two carboxylic acids, which are conserved within each glycoside hydrolase family, and proceeds through oxocarbonium ion-like transition states. Inverting glycosidases use a single displacement mechanism in which a water molecule directly displaces the aglycone through the direct involvement of two carboxylic amino acids of the enzyme. In this general mechanism, one of these carboxylic side chain acts as a general base while the other carboxylic side chain acts as a general acid. In a similar way, catalysis by retaining proceeds via a two-step double-displacement mechanism involving a glycosyl-enzyme intermediate. During the first step of this reaction (glycosylation), the acid–base catalyst protonates the glycosidic oxygen concomitantly with bond cleavage and the nucleophile attacks at the anomeric center of the substrate sugar to form a covalent glycosyl-enzyme intermediate. In the second step (deglycosylation), the acid–base catalytic group deprotonates the incoming water molecule, which then attacks at the anomeric center of the substrate while displacing the sugar (Davies *et al*., 1998; Zechel and Withers, 2000).

Synergy is observed when the amount of products formed by two or more enzymes acting together exceeds the arithmetic sum of the products formed by the action of each individual enzyme (Coughlan *et al*., 1993; Biely, 2003). Synergistic and cooperative effects among the xylanolytic enzymes enhance the susceptibility of the heteropolymeric xylan to be attacked by endoxylanases (de Vries *et al*., 2000). Dekker (1983) suggested that the synergistic action of β-xylosidases and xylanases is required for enzymatic degradation of xylan to xylose. Further studies have also verified that both enzymes acting together enhance the release of reducing sugars from arabinoxylan (Deshpande *et al*., 1986; Ximenes *et al*., 1996; Tuncer and Ball, 2003; Rasmussen *et al*., 2006; Yan et al., 2008). In such cases, β-xylosidases may be responsible for removing xylooligosaccharides, the product inhibition of xylanase, allowing a more efficient hydrolysis of xylan.

Heterosynergy is defined as the synergistic interaction between side-chain and main-chain cleaving enzymes. The presence of multiple extracellular xylan-degrading enzymes acting in synergism is often reported in aerobic fungi (Coughlan *et al*., 1993). Some fungi, such as *Penicillium capsulatum* and *Talaromyces emersonni* possess complete xylan degrading enzyme systems (Filho *et al*., 1991). Examples of heterosynergy between main-chain cleaving enzymes and α-arabinofuranosidase have been described in the literature (Poutanen, 1988; Ximenes *et al*., 1996). Tuncer and Ball (2003) suggested that β-xylosidases, α-L-arabinofuranosidases and xylanases, together, display a synergic action in xylan degradation. Endoxylanase should generate free chain ends upon which the β-xylosidase could act, while the debranching activity of α-L-arabinofuranosidase could remove substituent arabinose that may otherwise obstruct the progress of xylanases and β-xylosidases. The combination of the commercial multicomponent enzyme preparations Ultraflo L, from *Humicola insolens* and Celluclast 1.5 L, from *Trichoderma reesei* had positive synergism in complete degradation of water soluble and insoluble wheat arabinoxylan, due to interactions between arabinofuranosidase, endoxylanase and β-xylosidase activities present in both enzyme preparations (Sorensen *et al*., 2007).

β-xylosidases are active against p-nitrophenyl glucosides artificial substrates; most of them are very specific for xylopyranosides, such as p-nitrophenyl-β-D-xylopyranoside (Bhattacharyya *et al*., 1997; Saha, 2003b; Ito *et al*., 2003; Lembo *et al*., 2006). Others are able to cleave p-nitrophenyl-α-L-arabinofuranoside, p-nitrophenyl β-L-arabinopyranoside, p-nitrophenyl-β-D-galactopyranoside or p-nitrophenyl-α-D-glucopyranoside (Deleyn, 1982; Margolles-Clark *et al*., 1996; Ximenes *et al*., 1996; Peralta *et al*., 1997; Kimura *et al*., 1999;

Kitamoto *et al*., 1999; Kiss and Kiss, 2000; Ito et al., 2003; Zanoelo *et al*., 2004). Most of the purified enzymes reported were not able to degrade oat spelt xylan. Generally, β-xylosidases exhibit little or no action against polymeric xylans (Coughlan *et al*., 1993, Polizeli *et al*., 2005; Katapodis *et al*., 2006). *T. reesei* β-xylosidase is an exception, because this multifunctional β-D-xylan hydrolase was able to form xylose from xylan (Herrmann *et al*., 1997).

True β-D-xylosidases release xylose from xylobiose and xylooligosaccharides, a typical feature of an exo-type xylanolytic enzyme (Herrmann *et al*., 1997). Thin layer chromatography and HPLC analyses of the end products resulting from the action of β-xylosidases on xylooligosaccharides mixtures revealed that the enzymes more commonly hydrolyzed xylobiose, xylotriose and xylotetraose. The *Aspergillus phoenicis* β-xylosidase hydrolyzed only up to xylotriose (Rizzatti *et al*., 2001), while the *Scytalidium thermophilum* β-xylosidase hydrolyzed up to xylotetraose (Zanoelo *et al*., 2004) and *Fusarium proliferatum* β-xylosidase hydrolyzed up to xylohexaose (Saha, 2003b). According to Yan *et al*. (2008), β-xylosidases that hydrolyze up to xylopentose seems to be more applicable for xylan saccharifications.

In some cases, the rate of xylose released from xylooligosaccharides by purified enzymes increases with chain length (Herrmann *et al*., 1997; Saha, 2001; Yan *et al*., 2008). The opposite was verified for *Aspergillus nidulans* and *Trichoderma viride* β-xylosidases (Kumar and Ramón, 1996; Matsuo *et al*., 1998), while increasing in chain length did not affect the xylose rate release from xylooligosaccharides by *Sporotrichum thermophile* β-xylosidase (Katapodis *et al*., 2006).

PROPERTIES

Both fungi and bacteria with the ability to degrade xylans produce a multiplicity of enzymes, some belonging to the same functional class and plant polymer specificity (Uffen, 1997). Generally, filamentous fungi produce a multiplicity of β-xylosidases. Typical isoenzymes include β-xylosidases from *A. pulverulentus*, *A. niger*, *Neocallimastrix patriciarum* and *P. herquei* that showed two forms (Sulistyo *et al*., 1995; van Peij *et al*., 1997; Zhu *et al*., 1997; Ito *et al*., 2003) while *Penicillium wortmanni* β-xylosidase showed four forms (Matsuo *et al*., 1987). These enzymes exhibit a diversity of physicochemical properties,

structures, specific activities, yields and particular specificity leading to an increasement in xylan degradation. According to Wong *et al*. (1988), isoenzymes may present different effectiveness in hydrolyzing xylobiose, substituted xylooligosaccharides, xylosyl substituents or oligosaccharides containing xylosyl and other residues. Multiple β-xylosidases can be distinct gene products or can be generated by differential post-translational processing, through glycosylation and/or proteolysis (Ito et *al*., 2003; Collins et al., 2005)

β-xylosidases isolated from different filamentous fungi differ markedly with respect of their physicochemical and biological properties (Table 01). Most of the purified fungal β-D-xylosidases exhibit molecular masses above 100 kDa while others exhibit lower molecular masses. Monomeric (de Almeida *et al*., 1995; Zanoelo *et al*., 2004; Rizzatti *et al*., 2005; Lembo *et al*., 2006, Yan *et al*., 2008) and dimeric forms (John *et al*., 1979; Sulistyo *et al*., 1995; Kumar and Ramón, 1996; Li *et al*., 2000; Eneyskaya *et al*., 2007) are widespread among β-xylosidases purified from these microorganisms. The carbohydrate content of most of them was estimated to be 10 to 30%. Higher carbohydrate content was verified in β-xylosidase from *Aspergillus versicolor* (47%) (Andrade *et al*., 2004), *Paecilomyces thermophila* (61.5%) (Yan *et al*., 2008) and *A. phoenicis* (43.5%) (Rizzatti *et al*., 2001). Bhattacharyya et al. (1997) identified the presence of cellobiose and sucrose associated with *Termitomyces clypeatus* β-xylosidase. These sugars did not affect catalytic activity, but promote further enzyme stability under denaturing conditions. However, some enzymes are not glycosylated including *Humicola grisea* var. *thermoidea* β-xylosidase (de Almeida *et al*., 1995).

Most of fungal β-xylosidases exhibited acidic isoelectric points in the range of 4.0-5. Only two pI values around neutrality were described (Zanoelo *et al*., 2004; Saha, 2003b). The β-xylosidase from *T. emersonni* had an unusual pI for filamentous fungi, at pH 8.9 (Tuohy *et al*., 1993).

These enzymes presented maximal activity in temperature from 30 ºC to 70ºC. In this respect, many mesophilic fungal β-xylosidases have been purified and characterized, but few β-xylosidases from thermophilic fungi have been studied. β-xylosidases from thermophilic strains, such as *A. niger*, *Aspergillus brasiliensis* (Pedersen *et al*., 2007) and from the thermotolerant fungus *A. phoenicis* (Rizzatti *et al*., 2001) presented optimal activity in higher temperatures around 75 ºC. Others from thermophilic strains include β-xylosidases from *Humicola grisea* var. *thermoidea* (de Almeida *et al*., 1995; Lembo *et al*., 2006), *S.*

thermophilum (Zanoelo *et al*., 2004), *S. thermophile* (Katapodis *et al*., 2006) and *P. thermophila* (Yan *et al*., 2008)

In recent years, interest in thermostable enzymes has increased since resistance to thermal inactivation has become a desirable property of the enzymes used in industrial processes. Thermostable enzymes are generally defined as those with optimal temperature catalysis above that for maximal growth of the microorganism or with exceptional stability above 50 ºC for an extended period (Gupta and Gupta, 1993). The thermal stability may also be related to half-life of the enzyme ($T_{1/2}$), the time required to reduce 50% of the activity at a stated temperature, considering that the inactivation follows first order kinetics (Stahl, 1993).

Many xylanolytic enzymes produced by mesophilic fungi lack thermostability, thereby limiting their industrial application. With some exceptions, the majority of microbial enzymes are used in industrial processes at temperatures ranging from 35 to 60 ºC (Walsh, 2002). Thermostable enzymes are interesting because elevation of the reaction temperature (up to a certain limit) generally increases the reaction rate and reduces the risk of microbial contamination (Collins *et al*., 2005). In addition, resistance against inactivation by detergents, organic solvents, chaotropic and oxidizing agents has been verified for these enzymes (Walsh, 2002).

Many fungal β-xylosidases produced by mesophilic fungi showing great thermostability were described. Among them can be cited β-xylosidases from *A. niger* which retained 99% and 44% of the original activity after 1 h of incubation at 60 ºC and 75ºC, respectively. *A. brasiliensis* β-xylosidase retained 62% of activity at 60 ºC and 38% at 75ºC, after 1 h of incubation (Pedersen *et al*., 2007). β-xylosidase from *Aspergillus oryzae* showed half life of 4 h a 60 ºC (Christov *et al*., 1999). The β-xylosidase from the thermotolerant *A. phoenicis* was completely stable up to 4 h at 60º or 21 days at room temperature (25 ºC) (Rizzatti *et al*., 2001), while the β-xylosidase from *Aspergillus fumigatus* retained 50% and 25% of the original activity after 3 h at 70 and 75 ºC, respectively (Lenartovicz *et al*., 2003). In this respect, strains of the genus *Aspergillus* have received particular attention due to their potential thermotolerance and production of thermostable enzymes (Castro *et al*., 1997).

Generally, most fungal β-xylosidases have optimal activity at acidic pH ranging from 4.0 to 6.0. However, other enzymes with optimal activity in more acid and neutral pH were already described. *Penicillium sclerotiorum* showed an unusual optimum pH of 2.5 (Knob and Carmona, 2009) while *S. thermophile* and *Talaromyces thermophilus* β-xylosidases presented optimal activity at pH 7.0 (Katapodis et al., 2006; Guerfali et al., 2008).

Relatively broad substrate specificities are common among enzymes which are involved in the hydrolysis of hemicellulosic substrates. The spatial similarity between D-xylopyranose and L-arabinofuranose leads to bifunctional xylosidase-arabinosidase enzymes (Li *et al*., 2000; Mai *et al*., 2000). β-xylosidase with bifunctional activity of (aryl) xylosidase and (aryl) arabinosidase is of interest for the utilization in substituted xylans. Many fungal β-xylosidases with additional α-L-arabinofuranosidase activity have been reported (Deleyn *et al*., 1982; Herrmann *et al*., 1997; Ransom and Walton, 1997; Andrade *et al*., 2004; Katapodis *et al*., 2006; Eneyskaya *et al*., 2007; Yan *et al*., 2008). Other enzymes showed low levels of β-glucosidase (Ziie *et al*., 1985; Andrade *et al*., 2004; Zanoelo *et al*., 2004) and xylanase activity (Herrmann *et al*., 1997; Matsuo *et al*., 1998), associated with β-xylosidase activity.

REGULATION OF β-XYLOSIDASE GENE EXPRESSION

Molecular mechanisms that control the regulation of xylanolitic gene expression are still poorly understood. Studies with different carbon sources and growth conditions for enzyme production have been carried out for its elucidation. Cell-wall degrading enzymes complete expression by fungi depends on mechanisms associated with the glucose regulation pathway. Studies with *Aspergillus* and *Trichoderma* spp. at cellular and molecular levels indicate that β-xylosidase genes expression is regulated at the transcriptional level (Strauss *et al*., 1995; Ruijter *et al*., 1997; van Peij *et al*., 1997). In most of the fungi studied, xylanolytic enzymes expression is subject to specific induction in the presence of xylan or xylose and to carbon catabolite repression mediated by the repressor CreA (de Vries and Visser, 2001; van Peij *et al*., 2001, de Groot *et al*., 2003; Prathumpai *et al*., 2004). Regulation is also mediated by the transcriptional activator XlnR, which regulates the expression of a number of genes involved in xylan degradation, such as those encoding for β-xylosidase, xylanase, arabinoxylan arabinofuranohydrolase, α-glucuronidase, and D-xylose reductase (van Peij *et al*., 1998; Hasper *et al*., 2002; Stricker *et al*., 2008). The *xlnR* gene transcription is modulated by carbon sources and its repression is mediated by CreA (de Vries *et al*., 1999; Tamayo *et al*., 2008) Thus, xylanolytic genes transcription is regulated by the balance between the transcription of the factor XlnR and the CreA repressor (Tamayo *et al*., 2008).

Induction

Although xylose is the end product inhibitor of β-xylosidases (Deleyn *et al*., 1982), it can act as inducer of xylanolytic gene expression. The best yield of β-xylosidase on xylose was observed with *T. reesei* (Kristufek *et al*., 1995), *A. nidulans* (Perez-Gonzales *et al*., 1998) and *A. versicolor* (Andrade *et al*., 2004). In many cases, in addition to other carbon source, the presence of xylose is required to induce β-xylosidase gene expression. *Trichoderma koningii* β-xylosidase was induced in a medium containing 1% oat spelts xylan and 0.1% xylose. The presence of xylose unequivocally enhanced the induction of β-xylosidase (Li *et al.*, 2000). The studies with *T. ressei* suggested that a low supply of xylose triggers β-xylosidase induction (Kristufek *et al*., 1995). In *A. niger*, the expression of *xlnR* is not sufficient for induction of genes encoding the xylanolytic complex. The presence of xylose is absolutely required (Tamayo *et al*., 2008).

In other enzyme systems, disaccharides or high molecular weight substrates have been found to be the best inducers of this enzyme (Rajoka *et al*., 1997). However, in some cases, higher levels of xylanolytic enzymes can be achieved in the presence of substrates derived from xylan (Biely, 1985; Prade, 1995). According to Kulkarni *et al*. (1999), xylanolytic activity is inducible and substrates derived from xylan play an important role in the induction of these enzymes. Xylan was the best β-xylosidase inducer for various filamentous fungi (Ito *et al*., 2003; Rahman *et al*., 2003; Krogh *et al*., 2004). The expression of *xlnD* of *A. nidulans* was specifically induced by oat spelt xylan, as well as xylose (Perez-Gonzalez *et al*., 1998). The inductive effect of xylan derived substrates seems to be dependent on their chemical composition and structure (Saraswat and Bisaria, 1997). Conversely, for several species of filamentous fungi, lignocellulosic materials appear to be better substrates than xylan and xylose for xylanolytic enzymes production (Haltrich *et al*., 1996).

β-D-xylopyranosyl residues can also act as an inducer of the xylanolytic complex (Reese *et al*., 1973; Rizzatti *et al*., 2001). β-methylxyloside, a synthetic structural xylobiose analogue has also been used as a β-xylosidase inducer (Saraswat *et al*., 1997). β-xylosidases are also induced by alcohols, with a dependency on the glycerol concentration (Ito *et al*., 2003). Some enzymes are induced by xylitol while for others, xylitol acts as non-inducing carbon source (Kumar and Ramón, 1996). The stabilizing effect of polyols depends on hydrophilic and hydrophobic characteristics of the enzyme; polyols have the capability to

form hydrogen bonds that are important for native conformation maintenance and for protein stabilization.

For a particular microorganism, the expression of β-xylosidases may be achieved by different inducers. For example, the carbon sources known to induce the *A. niger* xylanolytic system were xylan, xylobiose and D-xylose, the latter two formed as a result of endo-xylanase and β-xylosidase activity (de Graaff *et al*., 1994). The *xypI* gene expression, encoding a β-xylosidase in *Cochliobolus carbonum* was induced by xylose and, in addition to xylan, it could be induced in the presence of other polysaccharides, such as pectin and cellulose (Tonukari *et al*., 2002). *T. emersonii* β-xylosidase gene (*bxl1*) was induced by xylan, methyl-β-D-xylopyranoside and xylose at lower concentrations (Reen *et al*., 2003). Both inductive and repressive effects have been observed in other fungi (Li *et al*., 1994; Magnelli and Forchiassin, 1999; Ponce-Noyola and De La Torre, 2001).

Repression

Several studies demonstrated that β-xylosidase-encoding genes are subject to catabolic repression (Kulmburg *et al*., 1993; van Peij *et al*., 1997). Carbon catabolic repression in microorganisms controls the synthesis of a range of enzymes required for the utilization of less favored carbon source when more readily metabolizable carbon sources are available in the medium (Ronne, 1995). In the presence of these sugars, such as glucose or xylose, the genes are not expressed. The repression of xylan degrading enzymes by preferred carbon sources such as glucose is an efficient energy-conserving mechanism, once when glucose is abundant in the environment; the xylan degrading enzymes are unnecessary (Tonukari *et al*., 2002). Carbon catabolite repression alters transcription and it is regulated by CreA protein (catabolite repressible entities), a transcriptional repressor of glucose-repressible genes (de Vries *et al*., 1999). Catabolic repression plays an important role in the regulation and secretion of inducible enzymes. At the molecular level, catabolic repression of β-xylosidases has been associated with the presence of binding sites for CreA in their promoters. Carbon catabolite repression mediated by CreA has also been observed in other fungal genes encoding for xylanolytic enzymes (de Graaff *et al*., 1994; Perez-Gonzalez *et al*., 1998; Mach *et al*., 1996)

In the presence of glucose, *A. niger* β-xylosidase gene (*xlnD*) expression is repressed. CreA binding sites were found upstream, in non-coding region, indicating that upstream

repressing sequences may directly control *xlnD* transcription (van Peij *et al*., 1997). The presence of six CreA binding sites in the upstream regulatory sequence (URS) of the *T. emersonni* β-xylosidase gene (*bxl1*) indicated that the observed repression by D-glucose may be mediated, at least partly, by this catabolite repressor (Reen *et al*., 2003). Recently, Tamayo et al. (2008) suggested that in *A. niger*, xylanolytic genes are subject to catabolic repression and provided evidence that CreA-mediated indirect repression occurs through repression of *xlnR*.

In spite of being an inducer of xylanolytic gene expression, xylose also represses the expression of xylanolytic genes. Depending on the concentration of xylose present in the medium, this sugar triggers CreA-mediated repression of gene expression. β-xylosidases produced by *T. emersonni* and *T. reesei* were inhibited by D-xylose, which is the principal end-product of hydrolysis of xylooligosaccharides, the natural substrate of these enzymes. The inhibition was competitive and the K_i was 1.3 and 2.4 mM, respectively, using PNPX as substrate (Poutanen and Pulls, 1988; Rasmussen *et al.*, 2006). Repression effect was also observed in *H. grisea* (de Almeida *et al*., 1995) and *A. phoenicis* (Rizatti *et al*., 2001). Conversely, several β-xylosidases have been proved to be xylose tolerant. *A. nidulans* β-xylosidase was only inhibited in the presence of xylose at 25 mM (Kumar and Ramón, 1996). *S. thermophilum* (Zanoelo *et al*., 2004) and *P. thermophila* (Yan *et al*., 2008) β-xylosidases demonstrated very high tolerance to xylose inhibition. Concentrations up to 200 mM for this product did not affect the former enzyme and the last one was 46.8% inhibited at 100 mM of xylose, with a K_i value of 139 mM. However, the majority of β-xylosidases presented a K_i for xylose ranging from 2 to 10 mM (Garcia-Campayo and Wood, 1993; Herrmann *et al*., 1997; Sunna and Antranikian, 1997; Saha, 2001; Saha, 2003b). In the saccharification process, β-xylosidase activity seems to play an important role by removing the end-product inhibition of endoxylanases (Sunna and Antranikian, 1997). For practical purposes, a xylose tolerant β-xylosidase is essential to maintain the efficiency of a developed process.

In some cases, β-xylosidase production is subjected to a form of carbon catabolite repression by glycerol and others alcohols (Ito *et al*., 2003; Katapodis *et al*., 2006). Besides, inductive/repressive effects of different nitrogen sources were verified. Rajoka (2007) suggests the existence of another regulatory mechanism for β-xylosidase synthesis in addition to induction. This regulation mechanism decreases the biosynthesis of β-xylosidase when an organism is grown on easily metabolizable substrates and different inorganic nitrogen sources.

CLASSIFICATION OF β-XYLOSIDASES

Numerous molecular studies on fungal β-xylosidases have been carried out through the last years and some filamentous fungal β-xylosidase genes have been isolated and sequenced (Table 02).

Classification of these enzymes based only on substrate specificity is difficult due to the existence of β-xylosidases with activity on different substrates. Currently, β-xylosidases are grouped in families, according to their amino acid sequence similarities. Once this classification based on primary structure which determines its tridimensional structure and molecular mechanism, members of these families presented similarity on structural and mechanistic characteristics, but differences in physicochemical properties, structure, mode of action and substrate specificities are verified. This system allowed the classification of glycosidases in general and became standardized for the classification of these enzymes. To date, according to Carbohydrate Active Enzymes database (CAZy), β-xylosidases are divided into families 3, 30, 39, 43, 52 and 54 of glycoside hydrolases (GHs) (Cantarel *et al*., 1999). However, filamentous fungal β-xylosidases have been described only for families 3, 43 and 54 (Ito *et al*., 2003; Eneyskaya *et al*., 2007, Wakiyama *et al*., 2008). Members of glycosylases families 3 and 54 operate with retention of the anomeric configuration, while GH43 family contains "inverting" glycoside hydrolases. Considering that protein fold is more conserved than their sequences, families with related three-dimensional structures are grouped into higher hierarchical levels, denominated clans (Davies and Henrissat, 1995).

Glycosyl hydrolases family 3

Family 3 is mainly composed of glucosidase (EC 3.2.1.21); xylan 1,4-xylosidase (EC 3.2.1.37); N-acetylhexosaminidase (EC 3.2.1.52); glucan 1,3-glucosidase (EC 3.2.1.58); glucan 1,4-glucosidase (EC 3.2.1.74); exo-1,3-1,4-glucanase (EC 3.2.1.-) and α-L-arabinofuranosidase (EC 3.2.1.55). This family was not related to any other GHs families.

The putative catalytic residues characteristic to GH3 are Glu and Asp, which are conserved within all glycosyl hydrolases of this family. Most of the cloned and sequenced

genes encoding family-3 GHs, were isolated from cellulolytic and xylanolytic microorganisms. Enzymes codified by these genes seem to be involved mainly in microbial macromolecules degradation (Faure, 2002). Many family-3 members exhibit a combination of different activities, exemplified by the frequent association of β-xylosidase and β-glucosidase activities, as previously mentioned. Such characteristic complicates the identification of their natural substrates as well as their denomination since the latter is based on the specificity of these enzymes. Transglycosylation activity has been reported in family-3 GHs (Herrmann *et al*., 1997; Kurakake *et al*., 2005, Wakiyama et al., 2008).

A. niger produce two β-xylosidases and both appear to be encoded by a single gene, *xlnD*, which has an open read frame (ORF) of 2412 bp and encodes a protein of 804 amino acids. The mature protein has 778 amino acids and a predicted molecular mass of 85 kDa. β-xylosidases I and II could be originated from different (N-terminal) processing, N-glycosylation events or other modifications, such as limited proteolysis (La Grange *et al*., 2001). The *A. awamori* K4 β-xylosidase gene (*Xaw1*) sequence was deduced by sequencing RT-PCR and PCR products. *Xaw1* has an ORF of 2.412 bp and the predicted peptide was 804 amino acids long, corresponding to a molecular weight of 87.15 kDa. The mature protein has 778 amino acids long with a molecular weight of 84.63 kDa. Amino acid sequence homology study revealed similarities to *A. niger* β-xylosidase gene (Kurakake *et al*., 2005).

The *A. nidulans* β-xylosidase gene (*xlnD*) was cloned and characterized. An ORF of 2.406 bp was revealed. The mature protein presented 785 amino acids long and a predicted molecular mass of 85.32 kDa. The deduced primary structure exhibited considerable similarity to the primary structure of the *A. niger* (64.3%) and *T. reesei* (61.9%) β-xylosidases (Perez-Gonzalez *et al*., 1998). The cloned *xylA* gene from *A. oryzae* encoding for β-xylosidase comprised 2.397 bp and encodes a protein consisting of 798 amino acids residues (86.47 kDa). The deduced amino acid sequence presented high similarities to *A. nidulans* XlnD (70%), *A. niger* XlnD (64%), and *T. reesei* BxII (63%). These highly conserved sequences appeared to be involved in the catalytic reaction, substrate binding or both (Kitamoto *et al*., 1999).

The gene coding for β-xylosidase, *bxl1*, has been cloned from the thermophilic filamentous fungus, *T. emersonii*. The gene *bxl1* consists of an ORF of 2.388 nucleotides which encodes a mature protein of 775 amino acids, with a predicted molecular mass of 86.8 kDa. The deduced amino acid sequence of the *T. emersonii bxll* gene product exhibited

considerable homology with the primary structures of the *A. niger*, *A. nidulans*, *A. oryzae*, and *T. reesei* β-xylosidase gene products and some β-glucosidases, which belong to the same glycosyl hydrolases family (Reen *et al*., 2003).

In addition, the *Aspergillus japonicus* β-xylosidase gene (*xylA*) was cloned and characterized. An open reading frame consisting of 2412 bp was revealed and it encoded a presumed a mature protein of 787 amino acids. The deduced amino acid sequence of *xlyA* gene revealed a high degree of identity to GH family-3 β-xylosidases and also showed significant levels of identity of amino acid sequence to the β-glucosidases belonging to GH family 3, indicating that the *A. japonicus* XylA belongs to GH family 3 (Wakiyama *et al*., 2008).

Glycosil hydrolase family 43

At the moment, glycoside hydrolases with variable activities belong to this family including: xylosidase (EC 3.2.1.37); 1,3-xylosidase (EC 3.2.1.-); L-arabinofuranosidase (EC 3.2.1.55); arabinanase (EC 3.2.1.99); xylanase (EC 3.2.1.8); galactan 1,3-galactosidase (EC 3.2.1.145).

Similarities on three-dimensional structure are found in families 43 and 62, thus both are grouped into clan GH-A. The topology observed for family 43 is a 5-fold-β-propeller. Enzymes in family 43 typically operate with inversion of the anomeric center (Cantarel *et al.*, 2009). Site-directed mutagenesis revealed a direct implication of Asp and Glu residues in substrate hydrolysis in members of this family (Reddy and Maley, 1996; Song and Jacques, 1999; Yanase *et al*., 2002). The plant-pathogenic fungus *C. carbonum* secretes one major β-xylosidase encoded by the gene *XYP1*. The mature protein has 328 amino acids long and a predicted molecular mass of 36.7 kDa. The primary sequence of Xyp1 is unrelated to any known eukaryotic β-xylosidase but has 35% overall identity to two bacterial bifunctional β-xylosidase/α-arabinosidase (Wegener *et al*., 1999).

The gene *s2* enconding S2 β-xylosidase in *P. herquei* was cloned and sequenciated. The *s2* gene consisted of 1.005 bp, encoding a mature protein of 335 amino acids. The predicted amino acid sequence presented high homology to that of *Bacteroides ovatus*, belonging to glycosidase family 43 (Ito *et al*., 2003). Family 43 xylosidase from fungi were

subdivided into two clusters and S2 and Xyp1 belonged to the same cluster. Despite only two β-xylosidase genes have been described and characterized to this family. Ito *et al*. (2003) related the existence of genes enconding family 43 β-xylosidase for *A. oryzae* and *Penicillium purpurogenum*, using primers designed based on the nucleotide sequence of *P. herquei s2* gene. The predicted amino acid sequences had high degrees of similarity to those of family 43 glycosidases and could be grouped to the same cluster of *s2* and *XYP1*. The authors suggested that xylosidases belonging to family 43 are distributed widely in fungi.

Glycosyl hydrolase family 54

At the moment, this family includes only two different glycosyl hydrolases activities: α-L-arabinofuranosidase (EC 3.2.1.55) and β-xylosidase (EC 3.2.1.37). GH54 remains unique from all existing clans. Based on protein structure and very preliminary mutagenic studies, Glu and Asp are candidates for the nucleophile and the general acid/base catalytic residues, respectively (Wan *et al*., 2007).

Only one enzyme with β-xylosidase activity has been included in this family. This bifunctional *T. koningii* β-xylosidase was encoded by the gene *xyl1* of 2.036 bp (Hseu *et al*., 1995). The *xyl1* nucleotide sequence revealed high homology with *A. nidulans abfB* and *T. reesei abf-1* arabinofuranosidase genes (Gielkens *et al*., 1999). More details about xyl1 remain unpublished.

Unclassified β-xylosidase

The *Magnaporthe grisea* sequenced genome presented an ORF of 546 bp encoding for a hypothetical protein with high similarity to fungal β-xylosidases (Dean *et al*., 2005, accession number XP_366835 in NCBI Protein Database). A recent study disclosed that the N terminal domain corresponded to the N terminal domain of glycosyl transferase family 32, which forms a five bladed beta propeller structure (accession number MGG_14303 in NCBI Protein Database). Members of family-32 GHs operate with retention of the anomeric configuration and Glu and Asp are the predicted catalytic residues (Cantarel *et al*., 2009). Up to now, information about *M. grisea* β-xylosidase is not available in CAZy database.

BIOTECHNOLOGICAL APPLICATIONS

β-xylosidase is a key enzyme in the xylanolytic system with a great potential in many biotechnological applications (Sunna and Antranikian, 1997). Over the years, β-xylosidases have been used in several conventional industrial processes, since their hydrolyzing and transglycosylating reactions are economically important. Enzyme preparations with β-xylosidases in their composition are commercially produced worldwide. Among the most important producers of these enzymes are *T. reesei* and *Humicola insolens*.

Many compounds such as oligosaccharides, glycoconjugates and neoglycoproteins can be synthesized through enzymatic synthesis, which posses many advantages over traditional organic synthesis (Ichikawa *et al*., 1992; Wong *et al*., 1994). Glycosides such as alkyl β-xylosides, ascorbid glucoside and α-arbutin, which have attracted special interest due to their physiological and physical usefulness can be prepared and synthesized by β-xylosidase transfer reaction (Sulistyo *et al*., 1995; Pan *et al*., 2001). Synthesis of β-xylanase substrates (Eneyskaya *et al*., 2003) and oligosaccharides modified with chromophoric or fluorogenic groups (Mala *et al*., 1999; Zeng *et al*., 2000) have also been obtained through β-xylosidase transxylosylation activity. Particularly, filamentous fungal β-xylosidases are more advantageous than those from other microorganisms and plants, due to their high stability and low commercial cost (Eneyskaya *et al*., 2003).

Another potential biotechnological application is for enzymatic saccharification of xylan rich agricultural wastes. Xylooligosaccharides produced by the lignocellulosic biomass degradation by diverse types of pretreatment need to be further hydrolyzed in order to be used by microorganisms for value-added products achievement. In this case, β-xylosidases are very useful in hydrolyzing these xylooligosaccharides to simple sugars (Saha, 2003a). D-xylose, the major sugar obtained by the enzymatic hydrolysis of xylan by the β-xylosidase activity, can be used as raw material for the bioethanol and xylitol production and as well as substrate for the production of various other chemicals (Skoog and Hahn-Hagerdal, 1988; Kuhad and Singh, 1993; Olsson and Hahn-Hagerdal, 1993; Sorensen *et al*., 2005). Xylose, xylose oligomers and xylitol are useful as sweeteners in foodstuffs, candies and medicaments, especially as a sugar substitute (Lachke, 2006).

Other potential applications of β-xylosidases, as well as other hemicellulases, include coffee processing, vegetable maceration, and preparation of high fiber baked goods (Coughlan *et al.*, 1993). β-xylosidases have been employed for hydrolysis of bitter compounds from grape fruit during juice extraction and liberation of aroma from grapes during wine making (Manzanares *et al*., 1999). In synergistic action with endo-β-xylanases and debranching enzymes have potential applications in the processing of food (Bajpai, 1997; La Grange *et al*., 2001). β-xylosidases or its products can be used as bread improvers and in the brewing industry. Much attention has also been devoted to xylan degrading enzymes in animal feed and the pulp and paper industries (Bajpai, 1999; Collins *et al*., 2005; Polizeli *et al*., 2005).

CONCLUDING REMARKS

Due to their biotechnological importance, significant progress has been made on the biochemistry and molecular biology of the β-xylosidases in recent years. Most of the studies performed so far have been concentrated on the screening, isolation, production, purification and characterization of β-xylosidases. These β-xylosidases, with different physical-chemical properties and their broad catalytic specificities contribute to our understanding towards xylan degradation. However, applied studies involving cooperativity and synergism among β-xylosidases and other xylanolytic enzymes in industrial process are lacking. Future studies on β-xylosidases should be conducted in this regard and devoted to the understanding and elucidation of the regulatory mechanism on the enzyme production at the molecular level. Up to now, little is known about the molecular mechanism by which low-molecular-mass compounds trigger xylanolytic system induction. Furthermore, new questions arise and require an explication, for example, the functional meaning of β-xylosidase multiplicity in a unique microorganism.

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TABLES

Table 01 – Some β-xylosidase properties from filamentous fungi

SPECIE		MW (kDa)	CARBOYDRATE CONTENT (%)	pI	OPTIMUM ACTIVITY		REFERENCE
					pH	TEMPERATURE (°C)	
Aspergillus. awamori K4		117	-	-	4.0	70	Kurakake et al., 1997
Aspergillus brasiliensis		-	-	-	5.0	75	Pedersen et al., 2007
Aspergillus carbonarius		100	-	4.4	4.0	60	Kiss and Kiss, 2000
Aspergillus fumigatus		-	-	-	5.4	70	Lenartovicz et al., 2003
Aspergillus japonicus		113.2	27.6	-	4.0	70	Wakiyama et al., 2008
Aspergillus japonicus		-	-	-	5.0	60	Pedersen et al., 2007
Aspergillus nidulans		180	-	-	5.0	50	Kumar and Ramon, 1996
Aspergillus niger		78	-	-	6.5	42	John et al., 1979
Aspergillus niger		-	-	-	5.0	75	Pedersen et al., 2007
Aspergillus. oryzae		-	-	-	4.0	60	Kitamoto et al., 1999
Aspergillus phoenicis		132	43.5	3.7	4.0-4.5	75	Rizzatti et al., 2001
Aspergillus pulverulentus	Xyl I	180	-	4.7	2.5-3.5	60	Sulistyo et al., 1995
	Xyl II	190		3.5	4.0-5.0	60	
Aspergillus sp.	I	-	-	-	6.5	80	Castro et al., 1997
	II				4.3	50	
Aspergillus versicolor	I	60	21	5.6	6.0	40	Andrade et al., 2004
	II	100	47	6.5	6.0	45	
Cochliobolus carbonum		42	-	-	5.5-6.5	37	Ransom and Walton, 1997
Fusarium proliferatum		91.2	-	-	4.5	60	Saha et al., 2003b
Fusarium verticillioides		94.5	-	7.8	4.5	65	Saha et al., 2001
Humicola grisea var. *thermoidea*		43	Not glycosylated	-	5.0	60	de Almeida et al., 1995
Humicola grisea var. *thermoidea*		50	-	-	6.5	55	Lembo et al., 2006
Neocallimastix frontalis		180	-	4.35	6.5	35	Hebrau and Fevre, 1990
Paecilomyces thermophila		53.5	61.5	-	6.5	55	Yan et al., 2008
Penicillium herquei	S1	103.7	-	-	4.0	50	Ito et al., 2003
	S2	37.5			6.5	30	

Penicillium sclerotiorum		-	-	-	2.5	60	Knob and Carmona, 2009
Penicillium. sp. AHT-1		-	-	-	6.0	50	Rahman et al., 2003
Penicillium. wortmanni QM 7322		-	-	5.0	3.3-4.0	-	Deleyn and Claeyssens, 1978
Penicillium. wortmanni IFO 7237	I	110	-	3.7	3.0-4.5	55-65	Matsuo et al., 1987
	II	195		4.3			
	III	210		4.6			
	IV	180		4.8			
Scytalidium thermophilum		45	12	7.1	5.0	60	Zanoelo et al., 2004
Sporotrichum thermophile		45	-	4.2	7.0	50	Katapodis et al., 2006
Talaromyces emersonii		-	-	-	3.0-3.5	60	Rasmussem et al., 2006
Talaromyces thermophilus		97	-	-	7.0	50	Guerfali et al., 2008
Termitomyces clypeatus		94	-	-	5.0	60	Bhattacharyya et al., 1997
Trichoderma harzianum		60	Small amount		4.0-4.5	70	Ximenes et al., 1996
Trichoderma koningii G-39		104	-	4.6	3.5-4.0	55-60	Li et al., 2000
Trichoderma reesei		100	-	4.7	4.0	60	Hermann et al., 1997
Trichoderma reesei			-	-	3.0-3.5	60	Rasmussem et al., 2006

Table 02 – Sequecend β-xylosidase genes from filamentous fungi

SPECIE	GENE	GLYCOSYL HYDROLASE FAMILY	REFERENCE
Aspergillus awamori K4	*Xaw1*	3	Kurakake *et al*., 2005
Aspergillus japonicus	*xylA*	3	Wakiyama et al., 2008
Aspergillus. nidulans	*xlnD*	3	Perez-Gonzalez *et al*., 1998
Aspergillus niger	*xlnD*	3	La Grange *et al*., 2001
Aspergillus oryzae	*xylA*	3	Kitamoto *et al*., 1999
Cochliobolus carbonum	*xyp1*	43	Wegener *et al*., 1999
Penicillium herquei	*s2*	43	Ito *et al*., 2003
Trichoderma emersonii	*bxl1*	3	Reen *et al*., 2003
Trichoderma koningii	*xyl1*	54	Hseu *et al*., 1995; Gielkens *et al*., 1999

FIGURE

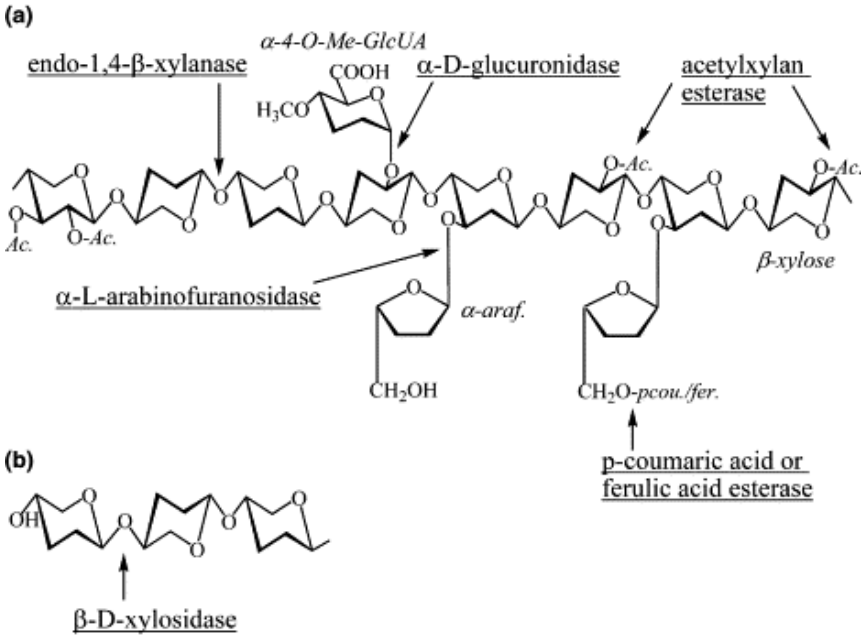

Fig. 1. (a) Structure of xylan and the sites of its attack by xylanolytic enzymes. The xylan main chain is composed of 1,4- β-linked xylose residues. Ac., Acetyl group; α-araf., α-arabinofuranose; α-4-*O*-Me-GlcUA, α-4-*O*-methylglucuronic acid; pcou., *p*-coumaric acid; fer., ferulic acid. (b) Action of β-xylosidase on xylooligossacharides. Captured from Collins et al., 2005.

CAPÍTULO 2:

Xylanase production by *Penicillium sclerotiorum* and its characterization

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ABSTRACT

Recently, xylanases have expanded their use in many processing industries, such as pulp and paper, food and textile. The production of xylanase by the fungus *Penicillium sclerotiorum* under submerse cultivation was investigated. Oat spelts xylan and wheat bran were the best inducers of xylanase activity. Optimal xylanase production was obtained in liquid Vogel medium, pH 6.5, at 30 ºC, under stationary condition during 8 days. Higher level of specific activity was obtained in 5 days of cultivation. The temperature for optimum activity was 50 ºC and optimum pH 4.5. The enzyme was stable at 40 ºC, with a half-life of 72 min. and when it was incubated at 45 and 50 ºC, the half-life was 8 min and shorter than 4 min, respectively. High pH stability was verified from pH 3.5 to 7.5. This enzyme showed interesting characteristics for some process, such as pulp and paper industry, because *P. sclerotiorum* produces low cellulase level, as well as in food industries.

Key words: Biochemical properties, *Penicillium sclerotiorum*, enzyme production, xylanase.

INTRODUCTION

Xylan, after cellulose, is the most abundant polysaccharide present in wood, agricultural and several agro-industrial wastes. This complex heteropolysaccharide consists of a main chain of 1,4-β-D-xylose monomers containing different substituents or ramifications [1, 2]. The substituents including arabinofuranosyl, glucuronyl and acetyl groups show a pronounced influence on its chemical and structural properties, and also on the enzymatic degradability of xylan in lignocelluloses [3, 4].

In nature, xylan is completely hydrolyzed to monosaccharides by the synergistic action of different enzymes [2]. The xylan-degrading system include endo-1,4-xylanases (1,4-β-xylan xylanohydrolase; EC 3.2.1.8), which release long and short xylo-oligosaccharides, or those that only attack longer chains and β-D-xylosidase (1,4-β-xylan xylohidrolase; EC 3.2.1.3.7), which remove D-xylose residues from short xylo-oligosaccharides [5, 6]. Enzymes such as α-arabinosidase, β-glucuronidase, ferulic acid esterase and acetyl xylan esterase are very important for the removal of side chain groups from xylan, especially when the fragments of the cleaved heteroxylans present high proportions of branched substituents [7].

Xylanases are produced by many different fungi and bacteria. From an industrial point of view, filamentous fungi are interesting producers of these enzymes due to xylanases releasing and their easy cultivation [8, 9]. These enzymes are commercially used in the pulp and paper, food, beverage, textile and animal feed industries [1, 10]. Moreover, xylanases show immense potential for increasing the production of several valuable products like xylitol and ethanol in a most economic way [11]. Most commercial xylanases are produced by *Trichoderma*, *Bacillus*, *Aspergillus*, *Penicillium*, *Aureobasidium*, and *Talaromyces* sp. [10, 12].

The Penicillia are mostly saprophytic in nature, and numerous species are of particular value for humanity [13]. The production of xylanolytic enzymes by Penicillia has been explored in a number of species. According to Chávez *et al*. [14], Penicillia constitute a rich source of enzymes for the biodegradation of xylan. Among 80 strains isolated from Brazilian soil at the Ecological Station of Juréia-Itatins in the Mata Atlântica region, the strain of *Penicillium sclerotiorum* attracted attention by producing xylanase in high levels, with low cellulolytic activity. In this paper, we report the production and characterization of xylanase secreted by this fungus.

MATERIAL & METHODS

Organism and growth

P. sclerotiorum used in the present work is available in the Culture Collection of Environmental Studies Center - CEA/UNESP, Brazil. It was cultivated for conidia production on Vogel's solid medium [15] containing 1.5% (m/v) glucose and 1.5% (m/v) agar at 25 ºC for 7 days. Liquid cultures were prepared in the same medium containing 1% (w/v) of the carbon source mentioned and the pH was adjusted for each experiment. Erlenmeyer flasks (125 mL) containing 25 mL of medium were inoculated with 1.0 mL of spore suspension (5 x 10^7 spores/mL) and incubated at different conditions as indicated subsequently. All cultures were developed in duplicate and the results are presented through mean values.

Enzyme preparations and assays

Cultures were harvested by filtration. The filtrate was assayed for extracellular activity and protein. The mycelium was washed with distilled and sterilized water, frozen and ground with sand in 50 mM sodium phosphate buffer pH 6.0. The slurry was centrifuged at 3.900 *g* at 4 °C and the supernatant was used as intracellular enzymes source.

Xylanase activity was assayed at 50 °C using 1.0 % (w/v) birchwood xylan (Sigma) in 50 mM sodium phosphate buffer pH 6.0. The reducing sugars released were quantified by the dinitrosalicylic acid method [16], using xylose as standard. One unit of enzyme activity was defined as the enzyme amount that releases 1 μmol of reducing sugar per min. Specific activity was expressed as unit per milligram of protein.

Protein determination

Protein concentration was determined by the Lowry method [17] with bovine serum albumin as standard.

Enzyme production on different carbon sources

The Vogel's medium was supplemented with various carbon sources at concentration of 1% (w/v). The inoculated flasks were incubated for 5 days at 28 ºC under agitation (120 rpm). Xylanase activity was determined in each case as described previously.

Effect of culture conditions, pH and temperature on xylanase production

The culture conditions influence on incubation period was studied under standing culture during 12 days and under shaking culture (120 rpm) during 8 days. The effect of initial pH on the enzyme production was analysed from 2.5 to 8.5 and the temperature influence was verified from 15 to 30 °C.

Enzyme characterization

Optimum pH and temperature xylanase activity

Enzyme activity was measured at 50 ºC in different pH values by the use of McIlvaine buffer from 3.0 to 8.0. The optimum temperature was determined by the incubation of the reaction mixture from 15 to 70 ºC and optimum pH.

Stability of xylanase at different temperatures and pH

Crude enzyme preparation was dilluted in McIlvaine buffer (1:2) in a pH range from 3.0 to 8.0 and incubated at 4 ºC for 24 h. The crude extract was incubated at 40, 45 and 50 °C in optimum pH determined above for different periods. The residual activity was determined in each sample, at the pH and temperature optimum for the enzyme.

RESULTS & DISCUSSION

Influence of the carbon source on xylanase production

In order to induce xylanase production, different substrates as pure carbohydrates and some natural substrates were tested (Table 1). Among the pure carbohydrates used, only oat spelts xylan induced xylanase production. According to Kulkarni *et al*. [18], xylanase activity

is inducible and substrates from xylan play an important role in xylanase induction. For others species of genus *Penicillium*, xylan also showed to be the best inducer [19, 20]. Enzyme activity was not detected with glucose, xylose, maltose, celobiose and sucrose as carbon source (Table 1), showing that this enzyme is non-inducible with these easily metabolizable sugars. Besides, *P. sclerotiorum* xylanase synthesis also can be affected by carbon catabolite repression, as verified in other filamentous fungi [21].

Among the agricultural and agro-industrial wastes, wheat bran showed to be the best inducer for xylanase production by *P. sclerotiorum* as for units of activity per volume and for specific activity as well. *Penicillium expansum*, *Penicillium* sp. ZH-30 and *Penicillium chrysogenum* xylanases also were induced by this carbon source [22-24]. Wheat bran induced very similar values of xylanolytic activity per volume as those obtained with oat spelts xylan. Carmona *et al*. [25] observed the same result in liquid cultures of *Aspergillus versicolor*. However, in this work, higher specific activity was verified in the presence of xylan, due to the minor amount of proteins released in the medium, than those detected in the presence of wheat bran. Media with oat bran exhibited lower xylanase activity than that observed with wheat bran. A low level of xylanase activity was verified with corn cobs. Absent or no significant levels of xylanase activity were obtained in cultures supplemented with all others substrates tested, such as orange bagasse, citrus pectin and soybean meal. *P. sclerotiorum* was not able to grow in the presence of sugar-cane bagasse and carboximethylcellulose (CM-cellulose). Avicel, rice straw, corn cobs and soybean meal provided minimal fungal development. According to previously published work, *P. sclerotiorum* produce low levels of cellulase [26], what may explain the fact that this fungus could not grow in the presence of some cellulosic predominant sources. In such case, this enzyme can be applied in biotechnological processes where the presence of cellulose is undesired. In all cases the values of intracellular activity (data not shown) were lower than those obtained extracellularly, as observed in most of the xylanases reported in the literature [22, 25, 27]. Thus, the carbon source used in the subsequent experiments was oat spelts xylan.

Effects of culture conditions on xylanase production

In standing culture, with oat spelts xylan as carbon source, the highest extracellular xylanase production was obtained in 8 and 5 days old cultures (27.21 U/ml and 65.29 U/mg of

protein) (Fig. 1a). In shaking condition (Fig. 1b), those maxima were observed at 3.5 days, corresponding to the values of 13.82 U/ml and 47.84 U/mg of protein. The highest *P. sclerotiorum* growth, measured by the intracellular protein concentration, occurred at 5 days in standing culture and 3 days in shaking culture (Fig. 1). In shaking condition, xylanase was expressed during the exponential phase and in standing condition, xylanase was expressed during the stationary phase, reaching the decline phase. According to Kulkarni *et al*. [18], xylanases are usually expressed at the end of the exponential phase and the harvesting time is correlated to the medium under consideration.

Examination of the macroscopic morphology of the *P. sclerotiorum* mycelium in stationary and shaked cultures revealed that in the former, the hyphae formed a freely dispersed mycelia, whereas in the latter, pellets were formed. It is well known that fungal morphology is influenced by agitation and that the formation of many products depends on the morphological structure of the macroscopic growth of filamentous fungi [28, 29]. In some cases, pellets formation is a prerequisite for secondary metabolites production, as citric acid and some fungal enzymes, such as polygalacturonase, glucoamylase or α-glucosidase. In others, as well as in this study, freely dispersed mycelium is preferred to higher metabolite production [30, 31]. Such factors probably explain the better xylanase production in standing than in shaked culture. For this reason, the subsequent experiments were carried out under stationary condition.

Temperature and pH are important environmental parameters that determine growth rates of microorganisms and significantly affect the level of xylanases produced. The influence of pH culture on xylanase production during *P. sclerotiorum* cultivation is showed in the Figure 2a. Xylanase activity was detected in all pH evaluated. The highest activity was observed at initial pH 6.5, corresponding to the values of 16.93 U/ml and 55.76 U/mg of protein. With rare exceptions, xylanase production by filamentous fungi occurs in cultures with an initial pH under 7.0. *Penicillium purpurogenum* [32] and *Penicillium janthinellum* [33] presented highest levels of xylanolytic activity at pH 5.5 and *Penicillium* sp. ZH-30 [23] at pH 6.0.

P. sclerotiorum could grow in media with initial pH between 2.5 and 8.5 (Fig. 2a), with maximal growth in the range of 3.0 to 4.0. This result clearly indicates the acidophilic nature of this fungus.

The effect of temperature on xylanase production by *P. sclerotiorum* is presented in Figure 2b. The highest xylanase activity per unit volume was verified at 25 ºC, while the

maximum value of specific activity was obtained at 30 ºC, corresponding to 18.92 U/ml and 55.42 U/mg of protein, respectively. Milagres *et al*. [34] reported that 30 ºC is the best culture temperature for xylanase production by *P. janthinellum* FM-5 and Haas *et al*. [35] obtained highest xylanase activity from *P. chrysogenum* at 28 ºC.

The highest growth was verified at 20 °C, indicating the mesophilic character of this strain. Nevertheless, lower values of xylanase activity per volume unit were obtained at 20 º C. According to Aiba *et al*. [36], at lower temperature, the transport of substrate across the cells is decreased and lower yield of products are attained. *P. sclerotiorum* was not able to grow at 35 ºC, a particular feature of this fungal specie [37].

Properties of extracellular crude xylanase

This study revealed that the best pH for this xylanase activity was around 4.5 (Fig. 3a). Studies carried out with *Penicillium* spp. [34, 38] as well with others fungal species [39, 40] also concluded that the most suitable pH value for xylanase activity was within the acid region.

The optimum temperature for xylanase activity was 50 °C (Fig. 3b). Similarly, in other studies with *Penicillium* spp., it was concluded that the optimum temperature varied between 40 and 50 °C [20, 38]. Besides, others fungal xylanases show optimum temperature at 50 ºC [41, 42].

Thermal stability is an interesting enzymes property due to the great industrial importance [43]. Then, enzyme stability analyses were carried out. The crude xylanase from *P. sclerotiorum* was incubated without substrate at 40, 45 and 50 °C (Fig. 4a). The half-life ($T_{1/2}$) at 40 °C was 72 min. At 45 °C, $T_{1/2}$ was 8 min and at 50 °C it was shorter than 4 min. An increase of thermal stability would be interesting and could be achieved with directed-site mutagenesis.

The xylanase produced by *P. sclerotiorum* maintained its stability over a broad of pH evaluated (Fig. 4b). Less than 50% of activity was verified in pH 3.0 and 8.0, while high stability (above 50 %) was observed from 3.5 to 7.5, with two major peaks at pH 4.0 and 6.0.

The xylanase from *P. sclerotiorum* was a novel enzyme, being active at acidic pH with an optimum at 4.5 and was stable in acid and neutral pH range. It showed optimum activity at 50 ºC and moderate stability at 40 ºC. These are desirable properties for application in the pulp and paper, as well as in food industries.

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TABLES

Table 1. Influence of some pure carbohydrates on xylanase production by *P. sclerotiorum*.

Carbon source (1 % w/v)	**Intracellular protein (mg)**	**Enzymatic activity (U/ml)**	**Specific activity (U/mg protein)**
Glucose	1.58 ± 0.19	ND	ND
Xylose	1.30 ± 0.09	ND	ND
Maltose	1.09 ± 0.07	ND	ND
Lactose	0.13 ± 0.01	ND	ND
Sucrose	2.12 ± 0.05	ND	ND
Celobiose	1.44 ± 0.12	ND	ND
Avicel	0.04 ± 0.00	ND	ND
CM-cellulose	ND	ND	ND
Oat spelts xylan	0.47 ± 0.02	7.82 ± 0.25	24.51 ± 0.20

Average and standard deviation of two cultures; ND: not detectable.

Table 2. Effect of different agricultural and agro-industrial wastes on xylanase production by *P. sclerotiorum.*

Carbon source (1 % w/v)	**Intracellular protein (mg)**	**Enzymatic activity (U/ml)**	**Specific activity (U/mg protein)**
Sugar-cane bagasse	ND	ND	ND
Wheat bran	1.03 ± 0.15	7.50 ± 0.06	21.71 ± 0.6
Oat bran	0.80 ± 0.03	3.58 ± 0.05	19.51 ± 0.9
Rice straw	0.01 ± 0.00	ND	ND
Soybean meal	0.06 ± 0.00	ND	ND
Corn cobs	0.03 ± 0.00	0.89 ± 0.02	3.61 ± 0.16
Citrus pectin	0.59 ± 0.07	ND	ND
Orange bagasse	0.56 ± 0.04	ND	ND

Average and standard deviation of two cultures; ND: not detectable.

FIGURES

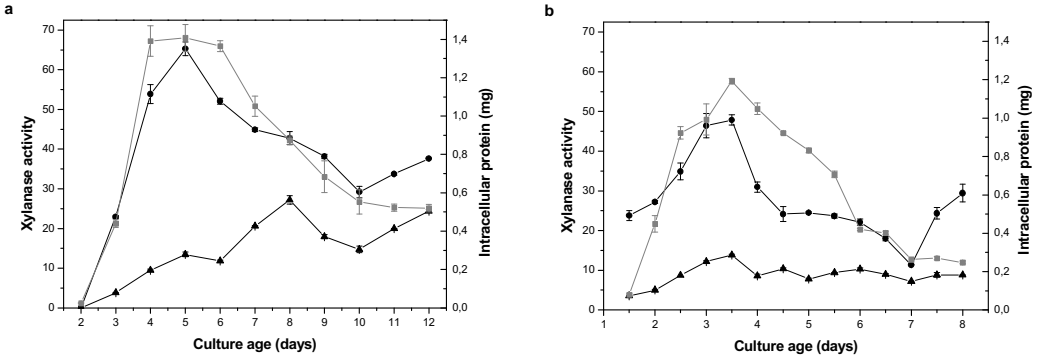

Figure. (1). Time-course of xylanase production by *P. sclerotiorum* in stationary (a) and shake culture at 120 rev min^{-1} (b). Culture conditions: Vogel medium with xylan 1 % (w/v), at 28 ^{o}C and pH 6.5. (▲) xylanase activity (U/ml), (●) specific xylanase activity (U/mg of protein); (■) intracellular protein (mg).

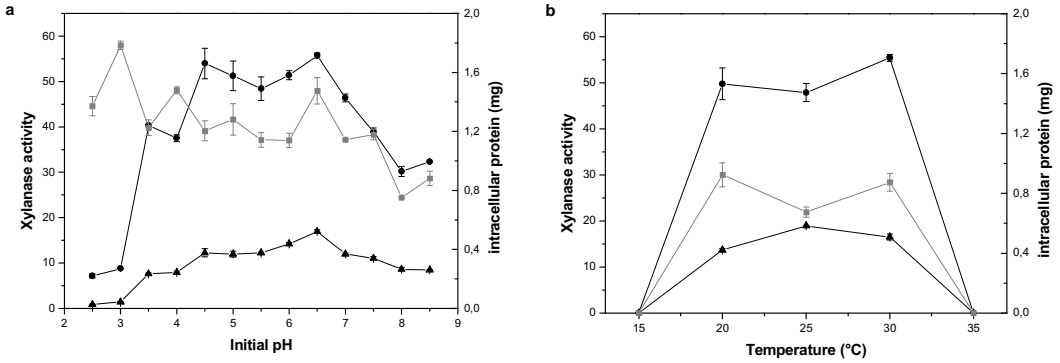

Figure. (2). Effect of initial pH (a) and temperature (b) on xylanase production by *P. sclerotiorum*. Culture conditions: Vogel medium with 1% xylan (w/v) under stationary condition for 5 days at 28 ºC (a) and pH 6.5 (b). (▲) xylanase activity (U/ml), (●) specific xylanase activity (U/mg of protein); (■) intracellular protein (mg).

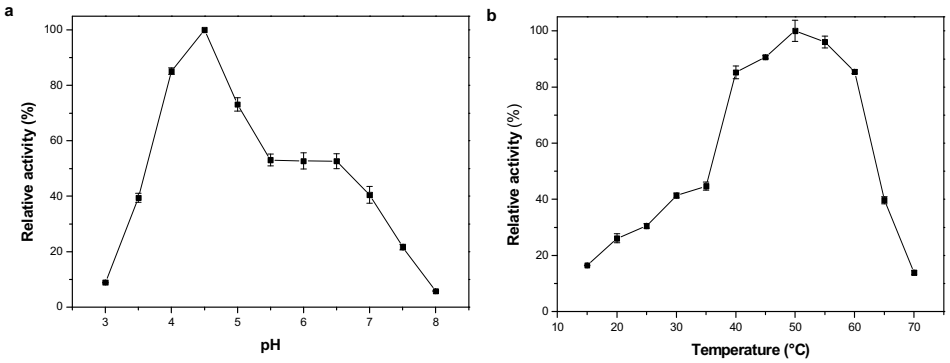

Figure. (3). Influence of pH (a) and temperature (b) on xylanase activity from *P. sclerotiorum*. Culture condition: Vogel medium with xylan 1 % (w/v) under stationary condition for 5 days, pH 6.5. Xylanase activity was assayed with McIlvaine buffer at 50°C (a) and with McIlvaine buffer pH 4.5 (b).

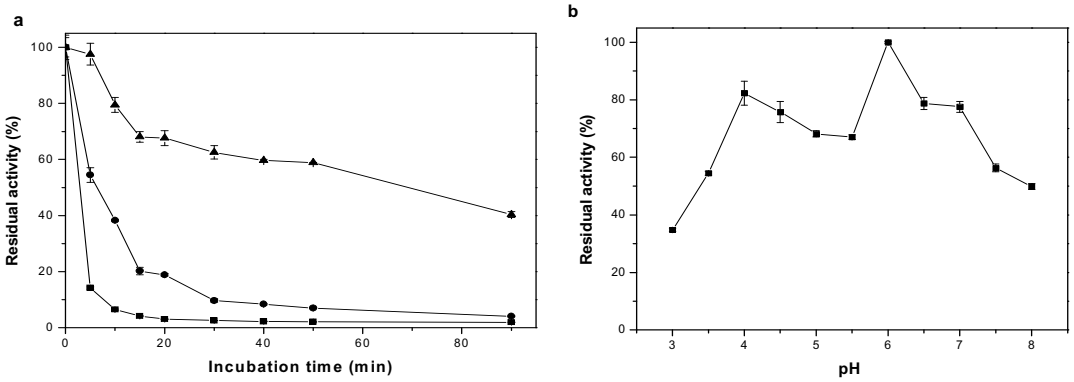

Figure (4). Thermal (a) and pH (b) stability of xylanase activity from *P. sclerotiorum*. (a) The crude filtrate was incubated at (▲) 40, (●) 45 and (■) 50 °C without substrate and the residual xylanase activity was assayed with McIlvaine buffer, pH 4.5, at 50 ºC. (b) The crude filtrate was incubated without substrate with McIlvaine buffer at 4 °C for 24 h and the residual xylanase activity was assayed with McIlvaine buffer, pH 4.5, at 50 ºC.

7. CAPÍTULO 3:

Cell-associated acid β-xylosidase production by *Penicillium sclerotiorum*

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ABSTRACT

In recent decades, β-xylosidases have been used in many processing industries. In this work, the study of xylosidase production by *Penicillium sclerotiorum* and its characterization are reported. Optimal production was obtained in medium supplemented with oat spelts xylan, pH 5.0, at 30 ºC, under stationary condition for six days. The optimum activity temperature was 60 ºC and unusual optimum pH 2.5. The enzyme was stable at 50 ºC and 55 ºC, with half-life of 240 and 232 min., respectively. High pH stability was verified from pH 2.0 to 4.0 and 7.5. The β-xylosidase was strongly inhibited by divalent cations, sensitive to denaturing agents SDS, EDTA and activated by thiol-containing reducing agents. The apparent V_{max} and K_m values was 0.48 μmole PNXP min^{-1} mg^{-1} protein and 0.75 mM, respectively. The enzyme was xylose tolerant with a K_i of 28.7. This enzyme presented interesting characteristics for biotechnological process such as animal feed, juice and wine industries.

Key words: Biochemical properties, enzyme characterization, enzyme production, *Penicillium sclerotiorum*, β-xylosidase.

Introduction

Xylan is the most abundant noncellulosic carbon source present in wood and agricultural residues, owing the fact that it is the major hemicellulosic polysaccharide in plant cell walls. This complex heteropolysaccharide consists of a linear backbone of β-1,4-linked D-xylopyranosyl units, which may have various substitutions and its full hydrolysis requires coordinated action of different enzyme activities (1,2). The most important of these are endo-*β*-1,4-xylanases (*β*-1,4-D-xylan xylanohydrolases, EC 3.2.1.8), which cleave internal glycoside bonds in the xylan backbone and *β*-xylosidases (*β*-1,4-D-xylan xylohydrolases, EC 3.2.1.37), which hydrolyze xylobiose and xylooligosaccharides to xylose, from the non-reducing end.

Interest in xylanolytic enzymes has been increasing, with a growing realization of their potential usefulness in biotechnology. Cellulase-free xylanases and β-xylosidases have a wide range of potential biotechnological applications in the pulp and paper, food and beverage, textile and agricultural industries (1,3).

The efficient production of fungal xylanolytic enzymes is dependent on culture parameters such as the nature of the inducing substrate, culture medium composition, temperature and pH. Thus, it is necessary to quantify the effects of these parameters to optimize the activity, rate and yield of xylanolytic production (4). Although considerable work has been carried out on xylan degradation, the role and specificity of the different components of the xylanolytic system remain unclear. A better understanding of the hydrolytic properties of these enzymes would be valuable to their industrial applications (5). Filamentous fungi are widely used as enzyme producers and are generally considered as more potent xylanolytic enzymes producers than bacteria and yeast (6, 7). β-xylosidases isolated from different filamentous fungi differ markedly with respect to their physicochemical and biological properties and mode of action. Most fungal β-xylosidases exhibited optimal activity at acidic pH ranging from 4.0 to 5.0 and the optimum temperature can vary from 40 to 80 ºC (7).

Fungi belonging to the genus *Penicillium* have a long history of use in the biotechnological enzyme production (8). According to Chávez et al. (9), *Penicillia* constitute a rich source of enzymes for xylan biodegradation. Among 80 strains isolated from Brazilian soil at the Ecological Station of Juréia-Itatins, SP, in the Mata Atlântica region, the strain of *Penicillium sclerotiorum* attracted attention for producing β-xylosidase activity at high levels

and low cellulolytic activity. In this paper, we report the production and characterization of the cell-associated β-xylosidase produced by this fungus.

Materials and Methods

Organism and growth

Penicillium sclerotiorum (*P. sclerotiorum*) used in the present work is available in the Culture Collection of Environmental Studies Center – CEA/UNESP, SP, Brazil. For conidia production it was cultivated on Vogel's solid medium (10) containing 1.5% (m/v) glucose and 1.5% (m/v) agar at 25 ºC for seven days. Liquid cultures were prepared in the same medium containing 1% (w/v) of the carbon source mentioned and the pH was adjusted for each experiment. Erlenmeyer flasks (125 ml) containing 25 mL of medium were inoculated with 1.0 ml of spore suspension (5 x 10^7 spores/ml) and incubated at different conditions as indicated subsequently.

Enzyme preparations and assays

Cultures were harvested by vacuum filtration. The filtrate was assayed for extracellular activity and protein. The mycelium was washed with distilled and sterilized water, frozen and ground with sand in 50 mM McIlvaine buffer pH 4.0. The suspension was centrifuged at 3.900 x *g* at 4 °C and the supernatant was used as a cell-associated enzyme and protein source. β-xylosidase activity was determined by measuring the p-nitrophenol released from p-nitrophenyl β-D-xylanopiranoside (PNPX) at 405 nm, according to Kersters–Hilderson et al. (11). A 0.2 ml solution of 0.25% PNPX (w/v) was preincubated for 5 min in McIlvaine buffer, pH 4.0, at 50 °C. For this solution, 0.3 ml of the properly diluted enzyme sample was added. After an appropriate period, aliquots of 0.1 ml were retired and the reaction was stooped by addition of 1.0 ml of saturated sodium tetraborate solution. One unit of enzyme activity was defined as the enzyme amount that releases 1 μmol of p-nitrophenol per ml, per minute of reaction. Specific activity was expressed as unit per milligram of protein. All enzyme assays were performed at least twice and the results are presented through mean values.

Protein determination

Protein concentration was determined by the Lowry method (12) with bovine serum albumin as standard.

Enzyme production on different carbon sources

The Vogel's liquid medium was supplemented with various carbon sources at a concentration of 1% (w/v). The inoculated flasks were incubated for five days at 28 ºC under agitation (120 rpm). β-xylosidase activity was determined in each case as described previously.

Effect of culture conditions, initial pH and temperature on β-xylosidase production

The incubation period influence on β-xylosidase production was studied under standing culture for 12 days and under shaking culture (120 rpm) for 8 days. The effect of initial pH on the enzyme production was assayed from 3.0 to 9.0 and the temperature influence was verified from 15 to 35 °C. The initial pH values were adjusted by addition of sodium hydroxide or phosphoric acid solutions.

Enzyme characterization

Exclusion molecular chromatography

To verify the presence or not of isoforms, the cell-associated enzyme preparation was dialyzed against 50 mM ammonium acetate buffer (pH 6.8) and then lyophilized and dissolved in a small volume of this buffer. This sample was applied to a column of Sephadex G-100 column equilibrated and eluted with the same buffer, flowing at 18 ml/h. Fractions (3 ml) were collected and tested for protein and β-xylosidase activity as previously.

Optimum pH and temperature β-xylosidase activity

β-xylosidase activity was measured at 50 ºC in different pH values using 0.05 M

glycine-HCl buffer for 2.0 and 2.5 and McIlvaine buffer from 3.0 to 8.0. The optimum temperature was determined by incubating the reaction mixture from 20 to 75 ºC in the optimum pH.

Stability of β-xylosidase at different temperature and pH

For pH stability assay, the enzyme preparation was diluted (1:2) in 0.05 M glycine-HCl buffer for 2.0 and 2.5 and in McIlvaine buffer in a pH range from 3.0 to 7.5 and incubated at 4 ºC for 24 h. To evaluate the thermal stability, the enzyme preparation was incubated at 50, 55 and 60 °C at optimum pH determined above for different periods. The residual activity was determined in each sample, at the optima pH and temperature.

Effect of various reagents

The effects of several metal ions and other reagents on the β-xylosidase activity were tested by measuring enzyme activity as described previously, in the presence of the individual reagents at concentrations of 2mM and 10mM. The relative activities were expressed as a percentage against the control.

Effect of substrate concentration

To determine the Michaelis–Menten constants for PNPX, an activity assay was performed with PNPX in concentrations between 0.5 and 15 mM at pH 2.5 and 60 ºC for 5 min. V_{max} and K_m values were calculated from Lineweaver–Burk plots using 'GraFit' software.

Tolerance of β-xylosidase to xylose

The extent of xylose inhibition was determined by incubating 10 µl enzyme preparation, 200 µl of 1mM PNPX dissolved in glycine-HCl buffer at pH 2.5 and 300µl of varying amounts of buffer and xylose with a xylose final concentration of 5–100mM, at 60 ºC for 5 min. The K_i was calculated using 'GraFit' software.

Results and Discussion

Influence of the carbon source on β-xylosidase production

Distinct substrates, such as pure carbohydrates and some natural lignocellulosic materials, were tested to induce β-xylosidase production (Table 1). Among the pure carbohydrates used, higher values of cell associated β-xylosidase activities were obtained with oat spelts xylan as carbon source. In general, higher levels of xylanolytic enzymes can be achieved with substrates derived from xylan (13,14). According to Kulkarni et al. (15), xylanolytic activity is inducible and substrates from xylan play an important role in the induction of these enzymes. Xylan also showed to be the best β-xylosidase inducer for other species of genus *Penicillium* (16-18). When *P. sclerotiorum* was cultivated with avicel and lactose as carbon source, enzyme activity was not detected. Xylose induced an intermediary level. Production of β-xylosidase on xylose is also reported by Andrade et al. (19) and Rajoka and Khan (20). In the presence of glucose, maltose, celobiose and sucrose, low levels of enzymatic activity were verified, when compared with oat spelts xylan values. These results indicate that this enzyme is inducible but can be affected by carbon catabolite repression, as verified in other filamentous fungi (21).

Among the agricultural and agro-industrial wastes, wheat bran showed to be the best inducer for β-xylosidase production by *P. sclerotiorum* for total units of activity and corn cobs for specific activity. However, these values were lower than the values obtained with oat spelts xylan. None or no significant levels of β-xylosidase activity were obtained in cultures supplemented with all other substrates tested, such as rice straw, soybean meal, corn cobs and sugarcane bagasse. In the production of xylanolytic enzymes by *Aspergillus tamari*, wheat bran was the best inducer among other lignocellulosic substrates, probably because it was able to minimize catabolic repression by glucose and other easily metabolizable sugars (22).

In this study, the extracellular activity expressed in total units corresponded only to 13.07% of cell-associated activity, in the presence of oat spelts xylan. The cell-associated β-xylosidase predominance in relation to extracellular form was also verified by Ito et al. (16) with *Penicillium herquei* and other fungi (23,24). Contrary to xylanases, which are always secreted by cells into the surrounding medium, β-xylosidases may be extracellular or cell-bound depending on the organism and the cultivation conditions (25).

Among the carbon sources tested, the best fungal growth was obtained with sucrose.

Glucose, celobiose, xylose and wheat bran were suitable for fungal development too. *P. sclerotiorum* was not able to grow in the presence of sugarcane bagasse and carboximethylcellulose (CM-cellulose). Minimal fungal development was provided by avicel, rice straw, corn cobs and soybean meal. Once *P. sclerotiorum* is a weak cellulase producer (26), this fungus could not grow in the presence of some cellulosic predominant sources. For this reason, this enzymatic complex can be applied to biotechnological processes in which the presence of cellulase is undesired. The carbon source used in the subsequent experiments was oat spelts xylan.

Effects of culture conditions on β-xylosidase production

Cultivation conditions are essential for the successful production of an enzyme, and optimization of parameters such as pH and temperature is important to develop the process. In standing culture (Figure 1a), with oat spelts xylan as carbon source, the highest β-xylosidase production was achieved in six-day-old cultures (11.00 U and 8.58 U/mg of protein). To specific activity, other expressive peak was observed on ninth day (7.93 U/mg of protein). In shaking condition (Figure 1b), maxima values for total units and specific activity were observed on 3.5 and 7-day-old cultures, corresponding to the values of 6.33 U and 9.61 U/mg of protein, respectively. The highest *P. sclerotiorum* growth, measured by the intracellular protein concentration, occurred on the fifth day in standing culture and on the third day in shaking culture (Figure 1). In shaking, as well as standing condition, β-xylosidases were expressed during the stationary phase, reaching the decline phase in the first case.

Higher values of β-xylosidase activity were obtained in standing rather than in shaked culture. Macroscopic morphology examination of the *P. sclerotiorum* mycelium in stationary and shaked cultures revealed that in the former, the hyphae formed a freely dispersed mycelia, whereas in the latter, pellets were formed. According to Braun and Vecht-Lifshitz (27), for producing fungal metabolites, the morphology varies from one product to another. In some cases, as well as in this study, freely dispersed mycelium is preferred to higher metabolite production (27,28). For this reason, the subsequent experiments were carried out under stationary condition.

Temperature and pH are some of the most important environmental factors for cell growth and product formation. The influence of pH culture on β-xylosidase production by *P. sclerotiorum* is shown in Figure 2a. β-xylosidase activity was detected in all pH evaluated.

The highest activity was observed at initial pH 5.0, corresponding to the values of 14.22 U and 11.83 U/mg of protein. In general, higher β-xylosidase activities were verified on cultures with pH adjusted from 4.0 to 5.0. According to Wainwright et al. (29), culture pH affects the spore surface properties, determining their dispersion and germination; therefore, it affects product formation. The favorable condition for xylanolytic enzyme production by filamentous fungus is slightly acidic pH (5 a 6.5) (30). *P. sclerotiorum* could grow in media with initial pH between 2.5 and 8.5 (Figure 2a), with maximal growth in 3.0. This result clearly indicates the acidophilic nature of this fungus.

The effect of temperature on β-xylosidase production by *P. sclerotiorum* is presented in Figure 2b. The highest β-xylosidase activity was verified at 30 ºC, corresponding to 17.53 U and 12.80 U/mg of protein. These results indicated that the enzyme production corresponded to the growth of the fungus. The optimum temperature for β-xylosidase production was similar to the optimum temperature for the growth of the fungus, corresponding to the temperature of the fungus natural habitat where it was initially isolated. This observation was in accordance with those reported by Kheng and Ibrahim (31). Moreover, the results obtained are related to those obtained by other authors who established that the best temperature range for β-xylosidase activity is between 20 ^{o}C and 35 ^{o}C (17,25).

The lowest values of β-xylosidase activity were verified at 15 and 20 °C. According to Rahman et al. (17), either very low or high temperatures inhibit fungal growth, leading to a decrease in enzyme synthesis. *P. sclerotiorum* was not able to grow at 35 ºC, a particular feature of this fungal species (32).

Properties of cell-associated β-xylosidase

Chromatographic analysis on Sephadex G-100 indicated the presence of only one β-xylosidase form (data not shown). The β-xylosidase activity was optimal at pH 2.5 (Figure 3a). Only *Aspergillus pulverulentus* showed a similar optimum pH value, between 2.5 and 3.5 (33). *Penicillium wortmanni* presented β-xylosidase with maximum activities at pH 3.3-4.0 (Deleyn et al. 1982). Lembo et al. (24) found a pH of 3.0 as optimum for a β-xylosidase produced by yeast *Aureobasidium* sp. However, β-xylosidases of fungal origin usually show optimal activity at pH values from 4.0 to 6.0 (16,19,34-38).

The optimum temperature for xylosidase activity was 60 °C (Figure 3b). Other studies with β-xylosidases from *Penicillium* spp. concluded that optimum temperature varied from 30

to 50 °C (9). Besides, other fungal β-xylosidases show the most favourable temperature at 55 (25), 60 (21,36,39) and 65 ºC (40).

Thermal and pH stability of β-xylosidase produced by *P. sclerotiorum* were investigated. The enzymatic preparation was incubated without substrate at 50, 55 and 60 °C (Figure 4a). This enzyme was stable at 50 °C and 55 ºC, retained 96% and 90% of its activity over 1 h at these temperatures, respectively. The half-life ($T_{1/2}$) values found were 240 min at 50 ºC and 232 min at 55 ºC. Nevertheless, at 60 °C the thermal stability rapidly decreased, presenting only 50% of activity after 20 min of incubation. Activity and stability at high temperatures are advantageous properties in these types of enzymes, since the most industrial processes where xylanolytic enzymes can be useful are carried out at high temperatures (41,42).

The β-xylosidase produced by *P. sclerotiorum* maintained its stability in very acid and neutral conditions (Figure 4b). High stability (above 80 %) was observed from 2.0 to 4.0 and 7.5, while less than 70% of activity was verified in pH 5.0–7.0.

The effect of various reagents on β-xylosidase activity was investigated (Table 2). β-xylosidase activity was assayed with the presence of several metals, sodium dodecylsufate (SDS), a metal chelator tetrasodium ethylenediaminetetraacetate (EDTA) and protein disulfide reducing agents dithio-***treitol*** (DTT) and β-mercaptoethanol, at 2mM and 10mM concentrations. The metal ions that inhibited the β-xylosidase were: strong inhibition, especially at 10mM, by all of divalent cations tested (Cu^{+2}, Zn^{+2}, Mn^{+2}, Ca^{+2}, Mg^{+2}, Co^{+2}, Pb^{+2}) and Na^{+}, except Ba^{+2}, which presented moderate effect (less than 20% of inhibition) and Hg^{+2}. It is remarkable that Hg^{+2} did not affect β-xylosidase activity. In general, this heavy metal is a strong inhibitor of xylan-degrading enzymes. The same effect was only observed in *Trichoderma harzianum* β-xylosidase (43). Especially the inhibition effects of Pb^{2+} for enzyme can occur with process of irreversible inhibition of enzyme.

The addition of EDTA affects β-xylosidase, suggesting that metals are needed for their enzymatic reactions. When SDS was tested, a total loss of the activity was observed, even at a low concentration (2mM), indicating that hydrophobic interactions are important in maintaining enzyme activity. The enzyme was remarkably stimulated by NH_4^+ and citrate at 10mM concentration. The increase in β-xylosidase activity indicates that NH_4^+ at the stated concentrations might be a cofactor for this enzyme. The requirement of monovalent cations for activities of a number of different enzymes has been reported in the literature. The malic enzyme from *Clostridium thermocellum* was similarly activated by NH_4^+. Increasing NH_4^+

concentration increased both enzyme activity and substrate affinity (44). Citrate can acts as chelant with a pronounced selectivity towards Ca^{+2}, Mg^{+2}, Fe^{+2} and other ions of similar size. Possibly, the increase in the enzymatic activity in the presence of citrate may be due to removal of any ion whose presence is detrimental to enzyme catalysis. However, NH_4^+ and citrate activations are properties not common to all previously described β-xylosidases. The stimulation of activity in the presence of reducing agents β-mercaptoethanol and DTT suggesting an influence of cysteine thiol groups in the catalysis of PNXP. These reducing agents bind to sulfhydryl groups preventing its oxidation, thus maintaining the enzyme structural integrity. *Aureobasidium* sp. β-xylosidase showed the same activation pattern (25), whereas *Paecilomyces thermophila* β-xylosidase was moderately inhibited by β-mercaptoethanol (93.7%) and did not affect by DTT (45).

Kinetic studies were conducted with the crude enzyme preparation and this can be justified since any process applications are unlikely to be based on purified enzymes. The rate dependence of the enzymatic reaction on the PNPX concentration at pH 2.5 and 60 ºC followed Michaelis–Menten Kinetics (data not shown). The apparent V_{max} and K_m values for the crude β-xylosidase determined by Lineweaver–Burk plots was 0.48 μmole PNXP min^{-1} mg^{-1} protein and 0.75 mM, respectively (Figure 5). These values are very similar to the ones obtained for purified *Fusarium proliferatum* β-xylosidase (39) and they are in accordance with the published values for the crude β-xylosidase activity of different filamentous fungi. The lower K_m value of *P. sclerotiorum* β-xylosidase shows that it has a substrate affinity higher than that of β-xylosidases from *Aspergillus nidulans*, *Scytalidium thermophilum* and *Paecilomyces thermophila* (45,46). However, β-xylosidases from *Neurospora crassa* (47) and *Aspergillus niger* (48) showed a greater affinity for this substrate than the enzyme from this study, with K_m values of 0.05mM and 0.22 mM, respectively.

Xylose is an end product inhibitor of β-xylosidases (49). For practical purposes, it is essential that β-xylosidase be xylose tolerant in order to make enzymatic saccharification of xylanolytic substrates an efficient process (38). So in order to determine the extent of xylose inhibition the enzyme preparation was incubated with PNPX, varying xylose concentrations (Figure 6). The enzyme was inhibited with a K_i of 28.7. Several β-xylosidases have proved to be xylose tolerant. *A. nidulans* β-xylosidase was only inhibited in the presence of xylose at 25 mM (45). Concentrations up to 200 mM for this product did not affect the *S. thermophilum* β-xylosidase (21). Nevertheless, the majority of β-xylosidases presented a K_i for xylose ranging from 2 mM to 10 mM (39,40,50,51).

Possible biotechnological applications

The β-xylosidase from *P. sclerotiorum* is a novel enzyme, activated by NH_4^+ and not inhibited by Hg^{+2}. Besides, this enzyme is active at very low pH, with an optimum at 2.5 and stable in acid pH range. These lasted characteristics make it potentially useful in some biotechnological processes. One of the specific applications is in animal feed industry. The addition of exogenous enzymes, including β-xylosidases to diets has significant potential to improve nutritive values of feed and animal performance (52-55). In recent years, only a limited number of commercial enzyme products have been brought out and the need for new enzyme products has increased (54). β-xylosidases with low pH optimum and broad pH stability would be the most suitable for using in animal feed, owing to extreme pH prevalent in the digestive tract. Other specific applications for *P. sclerotiorum* β-xylosidase are its use in clarification and maceration of juices and wines (13). One of the reasons why the use of the enzymes in these processes is limited is the physicochemical characteristics of must and wines, such as extremely acid pH (56). For these purposes it is crucial that enzymes be able to operate at very acidic conditions (57). Furthermore, *P. sclerotiorum* β-xylosidase could be of interest in winemaking, to promote the release of glycosides from monoterpenes and, thereby, increase the amount and quality of the flavour (58). Enzymes used in this application should be active and stable at low pH (59) because the usual pH range in winemaking is 2.5-3.8 (58).

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TABLES

Table 1. Influence of some pure carbohydrates and different agricultural and agro-industrial wastes on β-xylosidase production by *P. sclerotiorum*.

Carbon source (1 % w/v)	Intracellular protein (mg)	Enzymatic activity (U)	Specific activity (U/mg protein)
Pure carbohydrates			
Glucose	1.58	0.04	0.03
Xylose	1.30	0.78	0.60
Maltose	1.09	0.07	0.06
Lactose	0.13	0.01	0.07
Sucrose	2.12	0.06	0.03
Celobiose	1.44	0.04	0.02
Avicel	0.04	ND	ND
CM-cellulose	ND	ND	ND
Oat spelt xylan	0.47	4.22	8.96
Natural lignocellulosic materials			
Sugarcane bagasse	ND	ND	ND
Wheat bran	1.03	1.47	1.44
Oat bran	0.80	0.24	0.31
Rice straw	0.01	ND	ND
Soybean meal	0.06	ND	ND
Corn cobs	0.03	0.05	1.94
Citrus pectin	0.59	0.01	0.02
Orange bagasse	0.56	ND	ND

Average of two cultures; ND: not detectable.

Table 2. Effect of different substances on relative activity (%) of β-xylosidase from *P. sclerotiorum*.

Substance	Relative activity (%)	
	Concentration	
	2 mM	10 mM
Control	100	100
$CuCl_2$	73.7 ± 0.4	20.3 ± 0.4
$ZnSO_4$	70.4 ± 2.0	25.4 ± 1.1
$MnSO_4$	70.1 ± 1.6	20.3 ± 0.2
$BaCl_2$	90.5 ±1.3	89.3 ± 1.8
$CaCl_2$	72.0 ± 1.9	29.5 ± 1.4
NH_4Cl	102.0± 1.55	134.3 ± 1.7
NaCl	75.1 ± 1.3	20.4 ± 0.9
SDS	ND	ND
PMSF	92.0 ± 0.9	89.3 ± 0.5
$MgSO_4$	50.2 ± 1.7	20.3 ± 0.2
Sodium citrate	101.3± 1.6	152.0 ± 2.8
DTT	113.4 ± 1.2	134.4± 2.0
$CoCl_2$	67.4 ± 2.1	52.7 ± 1.8
$HgCl_2$	99.1 ± 0.5	98.8 ± 0.9
$Pb(CH_3COO)_2$	70.7 ± 2.5	59.0 ± 0.9
EDTA	89.0 ± 2.0	64.8 ± 1.0
β-mercaptoethanol	124.0 ± 2.0	136.0 ± 2.0

FIGURES

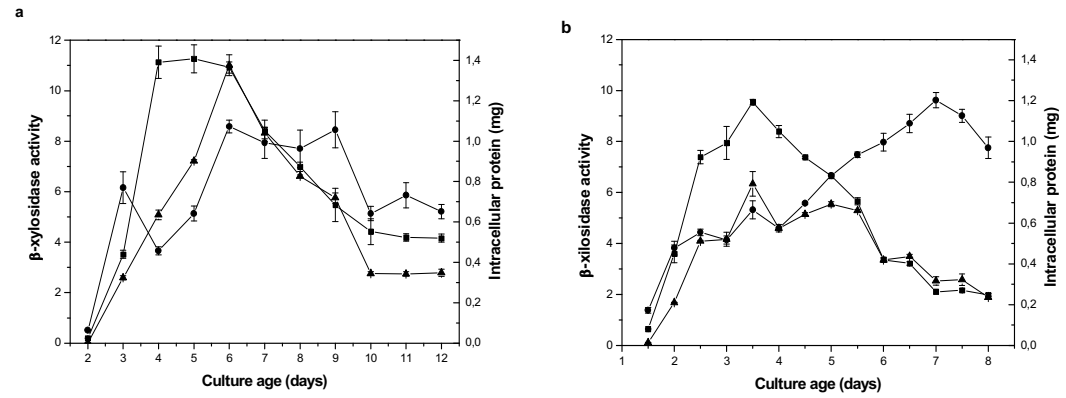

Figure 1. Time-course of β-xylosidase production by *P. sclerotiorum* in stationary (a) and shake culture at 120 rev min^{-1} (b). Culture conditions: Vogel medium with xylan 1 % (w/v), at 28 ^{o}C and pH 6.5. (▲)β-xylosidase activity (U), (●) specific β-xylosidase activity (U/mg of protein); (■) intracellular protein (mg)

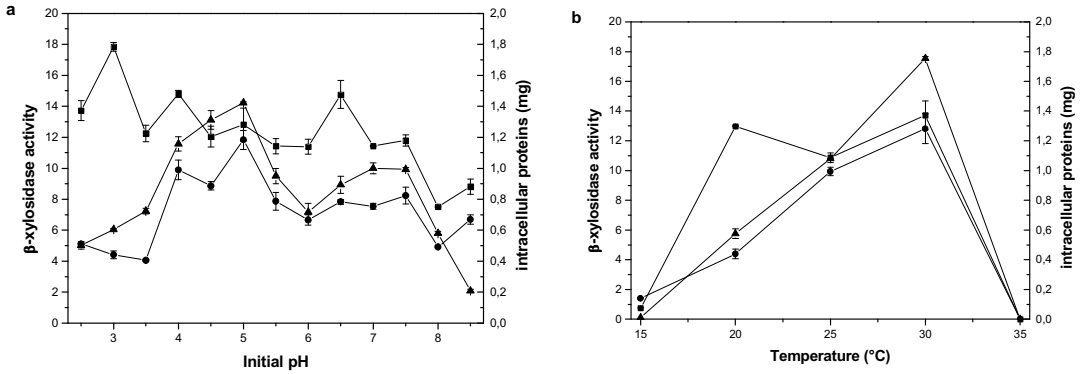

Figure 2. Effect of initial pH (a) and temperature (b) on β-xylosidase production by *P. sclerotiorum*. Culture conditions: Vogel medium with 1% xylan (w/v) under stationary condition for six days at 28 ºC (a) and pH 5,0 (b). (▲)β-xylosidase activity (U), (●) specific β-xylosidase activity (U/mg of protein); (■) intracellular protein (mg)

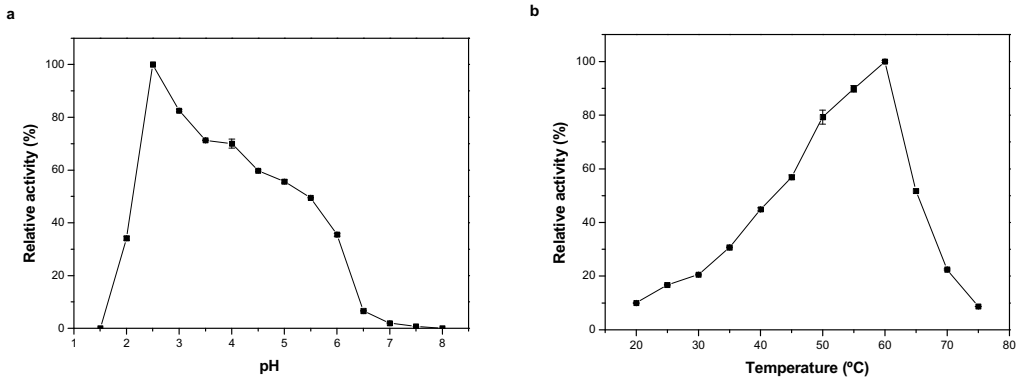

Figure 3. Influence of pH (a) and temperature (b) on β-xylosidase activity from *P. sclerotiorum*. Culture condition: Vogel medium with xylan 1 % (w/v) under stationary condition for six days, pH 5.0. β-xylosidase activity was assayed with glycine-HCl from pH 1.6 to 2.5 and McIlvaine buffer from 3.0 to 8.0, at 50°C (a) and with glycine-HCl buffer pH 2.5 (b).

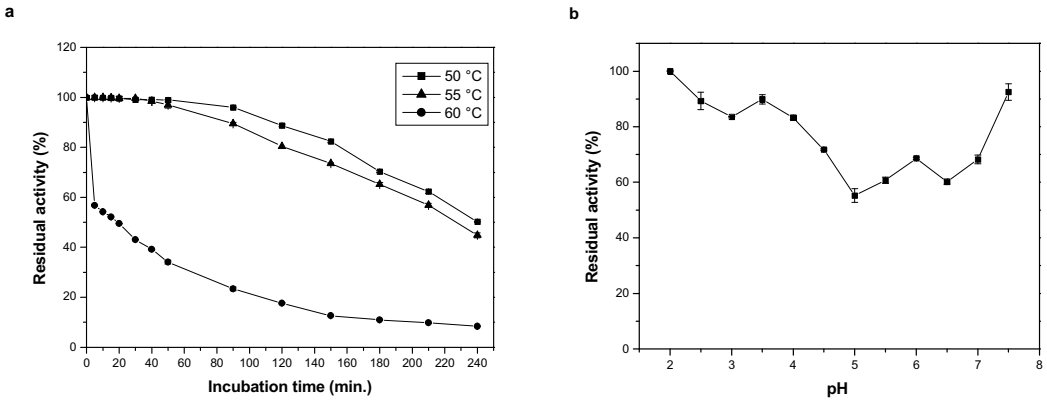

Figure 4. Thermal (a) and pH (b) stability of β-xylosidase activity from *P. sclerotiorum*. (a) The enzymatic preparation was incubated at (■) 50, (▲) 55 and (●) 60 °C without substrate (b) The enzymatic preparation was incubated without substrate with glycine-HCl buffer for pH 2.0 and 2.5 and McIlvaine buffer from pH 3.0 to 7.5 at 4 °C for 24 h. In both assays, the residual β-xylosidase activity was assayed with glycine-HCl buffer, pH 2.5, at 60 ºC.

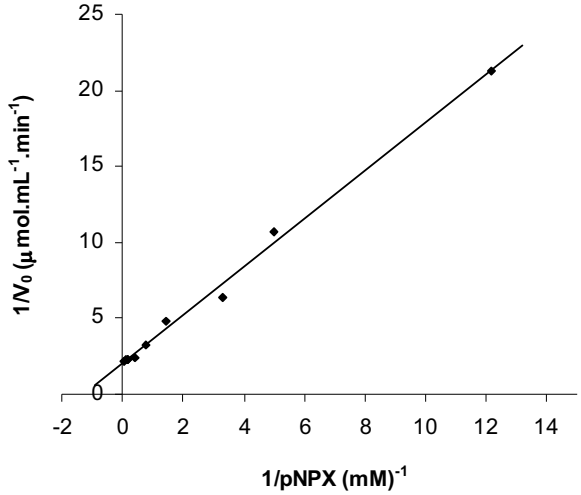

Figure 5. Lineweaver-Burk plots of the crude β-xylosidase activity. The reactions were monitored at 60 ºC and at pH 2.5 (glycine-HCl buffer). The data are presented as means for duplicate measurements.

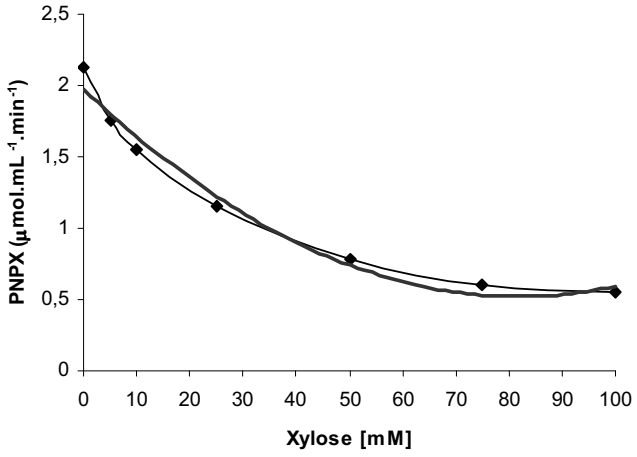

Figure 6. Inhibition of β-xylosidase by xylose. The enzyme preparation was incubated with PNPX, varying xylose concentrations (5–100mM), at 60 ºC for 5 min. The data are presented as means for duplicate measurements.

8. CAPÍTULO 4:

Purification and characterization of two extracellular xylanases from *Penicillium sclerotiorum*: a novel acid xylanase

KNOB, A.; CARMONA, E.C. **Applied Microbiology and Biotechnology**, 2009 (*in press*)
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Abstract

Two xylanases from the crude culture filtrate of *Penicillium sclerotiorum* were purified to homogeneity by a rapid and efficient procedure, using ion-exchange and molecular exclusion chromatography. Molecular masses estimated by SDS-PAGE were 23.9 and 33.1 kDa for xylanase I and II, respectively. The native enzymes molecular masses of 23.8 and 30.8 kDa were estimated for xylanase I and II, respectively, by molecular exclusion chromatography. Both enzymes are glycoproteins with optima temperature and pH of 50 ºC and pH 2.5 for xylanase I and 55 ºC and pH 4.5 for xylanase II. The reducing agents β-mercaptoethanol and DTT enhanced xylanases activities, while the ions Hg^{2+} and Cu^{2+}, as well the detergent SDS were strong inhibitors of both enzymes, but xylanase II was stimulated when incubated with Mn^{2+}. The K_m value of xylanase I for birchwood xylan and for oat spelt xylan were 6.5 mg mL^{-1} and 2.6 mg $mL^{-1,}$ respectively, whereas the K_m values of xylanase II for these substrates were 26.61 mg mL^{-1} and 23.45 mg mL^{-1}. The hydrolysis of oat spelt xylan by xylanase I released xylobiose and larger xylooligosaccharides while xylooligosacharides with a decreasing in polymerization degree up to xylotriose were observed by the action of xylanase II. The present study is among the first works to examine and describe an extracellular highly acidophilic xylanase, with an unusual optimum pH at 2.5. Previously, only one work described a xylanase with optimum pH 2.0. This novel xylanase showed interesting characteristics for biotechnological process such as feed and food industries.

Introduction

β-1,4-xylan is a major structural polysaccharide of plant cell walls being the second most prevalent in nature after cellulose. It is a heterogeneous polymer constituted primarily by a linear β-(1,4)-D-xylose backbone, which is partially acetylated and substituted in different degrees by a variety of side chains, mainly single α-D-glucuronosyl and α-L-arabinosyl units. Due to its structural complexity, several hydrolases are required for its complete degradation. The key enzyme in this process is endo-β-(1,4)-xylanase (EC 3.2.1.8), which cleaves the internal β-(1→4) bonds in the xylan backbone at nonmodified residues, yielding different chain length substituted xylooligosacharides [1-3].

Interest in xylanolytic enzymes has increased in recent years due to their potential application in biotechnology. Xylanases have been studied by their importance in several industrial processes, such as bioconversion of lignocellulosic materials into fermentative products, improvement of digestibility of animal feedstock, clarification of juices and facilitating the release of lignin from the pulp, thereby reducing the chlorine amount required for bleaching in pulp and paper industry [4-6]. In addition, they can be effectively used with cellulases to hydrolyze the lignocellulosic biomass generated which can be converted in bioethanol and xylitol [4, 7]. Xylanases are also employed for degumming of fibers such as flax, hemp, jute and ramie and in baking, to increase elasticity and strength of the dough leading an improvement of loaf volumes and texture of bread [4, 8].

Xylanases are produced by a variety of microorganisms, including filamentous fungi and bacteria, and their enzyme systems have been most widely studied [3, 9]. In many of these microorganisms multiple forms of xylanases has been observed. These enzymes may have diverse physico-chemical properties, structures, specific activities and yields, increasing the efficiency and extent of xylan hydrolysis. Fungal xylanases are more interesting from industrial point view because their extracellular activities are much higher than those from yeasts and bacteria [9, 10]. Species of Aspergilli, Penicillia and Tricodermii are examples of microorganisms which can produce xylanolytic isoenzymes. The Penicillia are mostly saprophytic in nature and numerous species are of particular value for humanity [11], many of them constitute a rich source of enzymes for xylan biodegradation [12].

Recently, we reported the best conditions for the xylanase production by a *Penicillium sclerotiorum* strain, isolated as a good xylanase producer [13]. In this paper, we described the purification and some properties of two extracellular xylanases produced by this fungus under

optimized culture conditions. The acidophilic and elevated pH stability of one purified xylanase in this work has potential applications in feed and food industries.

Materials and methods

Organism and growth

P. sclerotiorum used in the present work is available in the Culture Collection of Environmental Studies Center – CEA/UNESP, SP, Brazil. Conidia were obtained from cultures in Vogel solid medium [14] containing 1.5% (w/v) glucose and 1.5% (m/v) agar at 25 ºC for 7 days. Liquid cultures were prepared in the same medium with 1% (w/v) oat spelt xylan as carbon source and pH was adjusted to 6.5. Erlenmeyer flasks (125 mL) containing 25 mL of medium were inoculated with 1.0 mL of spore suspension containing 5 x 10^7 spores/mL and incubated at 30 ºC for 5 days in stationary condition. The mycelium was removed by vacuum filtration and the crude culture filtrate was used as a source of extracellular proteins

Enzyme assay

Crude and purified xylanases activities were determined at 50 ºC using 1.0 % (w/v) birchwood xylan (Sigma, ST Louis, MO) in McIlvaine buffer pH 4.5. This buffer is prepared from a mixture of 0.1 M citric acid and 0.2 M sodium monohydrogen phosphate. After 5 and 10 min of incubation, the reaction was interrupted by additon of 3,5-dinitrosalicylic acid (DNS) and the reducing sugars released were quantified [15], using xylose as standard. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugar per min, under assay conditions. Specific activity was expressed as unit per milligram of protein. All enzymatic assays were developed in triplicate and the results are presented through mean values.

Protein determination

Total protein was determined by Lowry's method [16], using bovine serum albumin (BSA) as standard.

Xylanases purificationIon-exchange chromatography on DEAE-Sephadex A-50

The crude filtrate of the fungal culture (200 mL) was dialyzed using a 12 kDa cellulosic membrane against 8 L of 0.05 M Tris-HCl buffer pH 9.0 for 8 h. The dialyzed crude enzyme was chromatographed on DEAE-Sephadex A-50 column (2.8 × 17.8 cm) equilibrated with the same buffer. Bounded proteins were eluted by a 0.0-0.5 M NaCl linear gradient, at a flow rate of 60 mL/h and 3.0 mL fractions were collected. Absorbance at 280 nm was measured and xylanase activity was determined. The fractions with significant xylanase activity were pooled and the samples were subjected to electrophoresis. All the purification steps were carried out at 4 °C.

Molecular exclusion chromatography

The samples corresponding to the retained and not retained fractions from ion-exchange column were further dialyzed against 8 L of 0.05 M ammonium acetate buffer pH 6.8 for 8 h with 4 changes, and then lyophilized and re-suspended in a small volume of this buffer. The samples were chromatographed on Sephadex G-75 column (2.5 x 64.0 cm) equilibrated and eluted with the same buffer, flowing at 18 mL/h. Fractions of 3 mL were collected and the protein content was recorded by reading absorbance at 280 nm and xylanase activity assayed as described previously. To determine xylanases molecular masses through gel filtration chromatography, the column was calibrated using blue dextran for the void volume determination and ribonuclease (15.4 kDa); chymotrypsin (25.0 kDa); ovalbumin (43.0 kDa) and bovine serum albumin (67.0 kDa) as standards. The molecular weights of xylanases were estimated from a regression curve (R^2 = 0.993), by plotting log of the molecular weights of the standards against the ratio between elution volumes of the standards and the void volume of the column.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a gradient of 8-18% (w/v) polyacrylamide according to Laemmli [17]. The resolved protein bands were visualized after staining with 0,1 % Coomassie brilliant blue R-250 dissolved in methanol, acetic acid and distillated water (4:1:5, v/v/v). The proteins phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin (SDS-LMW markers – Sigma, ST Louis, MO) were used to plot the standard curve log of molecular weight against relative mobility in the gel.

Purified xylanases characterization

Determination of carbohydrate concentration

Total carbohydrate was measured colorimetrically according to Dubois phenol-sulphuric acid method [18], with glucose as standard.

Temperature and pH optima, thermal and pH stability

The optimum temperatures were determined by performing the reaction at temperatures ranging from 15 to 70 ºC in McIlvaine buffer pH 4.5. To determine the optimum pH, the purified xylanases were assayed at 50 ºC or 55 ºC, in different pH values using 0.05 M glycine-HCl buffer for pH from 1.6 to 2.5 and McIlvaine buffer from 3.0 to 7.5.

For pH stability assays, the purified enzymes were diluted (1:2 v/v) in 0.05 M glycine-HCl buffer for pH 1.6 to 2.5 and in McIlvaine buffer for pH range from 3.0 to 7.5. The samples were incubated at 4 ºC for 24 h. After this period, the activities of xylanases were assayed under optimal conditions for each enzyme. To evaluate the thermal stability, the purified enzymes were incubated at different temperatures at the optimal pH determined above for different periods.

Effect of substances

The effect of metallic ions and other compounds on the activity of the purified xylanases were evaluated at concentrations of 2 mM and 10 mM. The residual activities were measured in relation to the control without substances by performing the enzyme assay at the optimal conditions for each enzyme.

Substrate specificity

Specificities of xylanases against birchwood xylan, oat spelt xylan, carboxymethyl cellulose and avicel were assayed. Substrate solutions of 1% (w/v) were prepared in a buffer of optimum pH activity for each enzyme.

Kinetic parameters

The enzymes were incubated with oat spelt and birchwood xylans, at concentrations between 4.0 and 30 mg/mL. The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated from the Lineweaver-Burk reciprocal plots, using 'GraFit' 5.0 software.

Mode of action

The products of enzymatic hydrolysis of 1% (w/v) oat spelt xylan reaction mixtures incubated in optimal conditions for the purified xylanases were examined by thin-layer chromatography (TLC) on silica-gel G-60 precoated plates (10 x 15 cm), as described by Fontana et al. [19]. After 10, 30, 120 and 1020 min of incubation at 50 ºC reaction mixtures were sampled, the enzyme activity was stopped by freezing, centrifugated and the samples were applied on TLC plates. The mobile phase was ethyl acetate/acetic acid/formic acid/distilled water (9:3:1:4; v/v/v/v). Plates were revealed by applying of 0.2% (w/v) orcinol in sulfuric acid/methanol (1:9, v/v).

Results and Discussion

Purification of xylanases

Conventional purification methods were effective to purify both xylanases from *P. sclerotiorum.* These enzymes were purified from the culture supernatant by chromatography on DEAE-Sephadex A-50 followed by Sephadex G-75 chromatography. After ion-exchange chromatography (Fig. 1) two protein peaks showing xylanase activity were obtained: xylanase I, not retained on DEAE-Sephadex A-50 resin, representing 63.8 % of the crude filtrate activity and xylanase II, recovered by elution with a NaCl gradient, corresponding to 24.3 % of the initial activity.

The pooled fractions corresponding to xylanase I and II were subsequently applied to molecular-exclusion chromatography. For both samples, only one protein peak with xylanase activity was observed (Fig. 2 and Fig. 3). The fractions corresponding to these peaks were collected and the samples showed electrophoretic homogeneity (Fig. 4). After purification, xylanase I exhibited a specific activity of 249.15 U mg^{-1} protein, 2.49 fold-purification and

recovery of 27.1 %. Xylanase II presented a specific activity of 240.89 U mg^{-1} protein, 2.41 fold-purification and recovery of 9.8 % (Table 1). Similarly to *P. sclerotiorum*, many microorganisms produce a multiplicity of xylanases to achieve effective hydrolysis of xylan [4].

The molecular weights estimated by SDS-PAGE were 23.9 and 33.1 kDa for xylanase I and II, respectively. Native enzyme molecular masses of 23.8 and 30.8 kDa were estimated for xylanase I and II, respectively, by molecular exclusion chromatography, showing monomeric forms. According to Törrönen and Rouvinen [20], microbial xylanases are usually monomeric proteins and the estimated values of their molecular weights are in agreement with those found for the catalytic domain of low molecular weight xylanases, belonging to family 11. Isozymes with different molecular masses were produced by the rumen fungus *Neocallimastix frontalis*, and molecular masses of xylanases I and II were 45 and 70 kDa, respectively [21]. Nair et al. [22] purified xylanase I and II from *Aspergillus sydowii*, with molecular masses of 20.1 and 43.0 kDa, respectively. The *Aspergillus giganteus* xylanases showed molecular masses of 21 and 24 kDa [23], while 19 and 32 kDa were the molecular masses estimated to xylanases from *Aspergillus versicolor* [24, 25].

The carbohydrate contents of purified enzymes were estimated to be 14.8 % for xylanase I and 65.1% for xylanase II. Glycosylation is a common feature among extracellular fungal xylanases [5]. High carbohydrate content was also verified in xylanases from *A. sydowii* (40.6 and 53.7 %) [22], *Aspergillus fumigatus* (46.4 and 68.0 %) [26] and *Aspergillus versicolor* (71.0 %) [24]. However, these carbohydrate contents are much higher than those observed for the xylanases from *Paecilomyces themophila* (21.0 %) [27], *Paecilomyces varioti* (4.5 %) [28] and a minor form of *Aspergillus versicolor* xylanase (14.1%) [25]. Glycosylation provides a pos-translational modification mechanism that modulates secreted enzymes. It has been show that glycosylation of xylanases might contribute to the stability of the protein conformation, thus increasing enzymatic activity [29].

Enzyme properties

Effects of pH and temperature, thermal and pH stabilities

The effects of temperature and pH on the activities of the purified xylanases were investigated (Fig. 5). Highest xylanase I activity was detected at 50 ºC compared to the 55 ºC observed for xylanase II. The optimal temperatures of the purified xylanases were similar to

other xylanases of fungal origin [4, 12]. Xylanase from *Aspergillus niger* presented optimal temperature at 50 ºC [30], while purified xylanases I and II from *Aspergillus caespitosus* exhibited the same optimum temperature of 50-55 ºC [31]. Optimum thermal parameters for the action of xylanases from *Fusarium verticilloides* [32] and *Penicillium citrinum* [33] were also identical to those determined for xylanase I. According to the thermostability assay (Fig. 6) the xylanase II in the present investigation was more stable than xylanase I. The half-life ($T_{1/2}$) exhibited by xylanase I and II at 50 ºC were 40 and 90 min, respectively. Xylanase II retaining almost 80 % of its activity after 90 min of incubation at 45 ºC, while xylanase I was stable at 40 ºC, maintaining approximately 85% of the initial activity after 90 min of incubation.

Xylanase I showed optimal activity at pH 2.5 and its activity was maintained over 80% at pH 1.6 to 3.0, while for xylanase II the optimum pH was 4.5, retaining more than 60% optimal activity in the pH range from 3.5 to 7.0 (Fig. 5b). *P. sclerotiorum* xylanase I showed activity at very low pH, one of the most acid described in literature, except for the xylanase from *Penicillium* sp. 40, with optimal activity at pH 2.0 [34]. *Laetiporus sulphureus* showed xylanase activity an optimum pH of 3.0 [35]. However, most of the reported xylanases had an optimum pH between 5.0 and 7.0 [36] and among the acidophilic xylanases, the majority of them showed high activity under slight acid conditions. In the pH range from 1.6 to 7.5, the purified xylanases exhibited distinct stability profiles (Fig. 7). Both enzymes exhibited high stability around its optimal pH. Xylanase I presented above 70% of its residual activity in all acid and neutral conditions evaluated. Xylanase II remained stable in pH ranging from 2.5 to 4.5, maintaining more than 80% of its activity in these conditions and showed more than 50 % of its activity in pH between 5.0 and 7.0. Microbial xylanases are usually stable over a wide pH range (3-10) and show optimum pH in the range 4.0-7.0 [2]. Studies with family 11 xylanases suggest a correlation between pH activity/stability and the number of salt bridges, with acidophilic xylanases presenting much less of these interactions than their alkalophilic homologs [37]. The optimum activity in very acid conditions and pH stability exhibited by xylanase I make its use attractive for some industrial applications, such as in feed and food industries.

Effect of substances

In order to verify the effect of substances on xylanases activities, the purified enzymes were incubated in the presence of several metallic ions, sodium dodecylsufate (SDS), tetrasodium ethylenediaminetetraacetate (EDTA), dithio-treitol (DTT), phenylmethylsulphonyl fluoride (PMSF) and β-mercaptoethanol, at 2 mM and 10 mM concentrations (Table 2). In general, the activities of both enzymes enhanced with increased concentration of the substances used. Hg^{2+} and Cu^{2+} were strong inhibitors of both xylanases, while Zn^{2+} had a moderate inhibitory effect on the xylanase I and II. Likewise, *Aspergillus fucuum*, *Aspergillus giganteus* and *Aspergillus versicolor* xylanases were inhibited by Hg^{2+} and Cu^{2+} [24, 25, 38]. The inhibition by Hg^{2+} seems to be a general property of xylanases, indicating the presence of thiol groups of cysteine residues in their active sites or around them [39]. Xylanase I activity remains unaltered in the presence of Na^{+}, Mn^{2+} and Co^{2+}, while the activity of xylanase II was not affected by Na^{+} and Mg^{2+}. Slight activation was observed for xylanase I and II in the presence of Ca^{2+} and sodium citrate. Additionally, xylanase I was activated by Ba^{2+} and NH_4^{+} and xylanase II activity was remarkably stimulated when incubated with Mn^{2+}.

EDTA, a metal chelator, decreased xylanases activities, indicating that the purified enzymes require metal ions for their actions. Total loss of activity was also observed for both enzymes in the presence of SDS, suggesting that hydrophobic interactions may be important in maintaining the structures of the enzymes. The reducing agents β-mercaptoethanol and DTT enhanced xylanases activities. The stimulation of the enzymatic activities in the presence of these thiol group protecting agents can be explained by preventing the oxidation of sulfidryl groups. Similarly, Fialho & Carmona [23], Dutta et al. [33] and Kang et al. [40] related the involvement of cysteine residues in the maintenance of tertiary structure of the active site in *Aspergillus giganteus*, *Acrophialophora nainana* and *Penicillium citrinum* xylanases.

Substrates specificity and kinetic studies

Specificity studies indicated that the xylanases did not hydrolyze avicel or carboxymethylcellulose, but acted only on xylans. Strong specificity toward birchwood and oat spelt xylans was also verified to xylanases from *Penicillium chrysogenum* [41] and *Aspergillus giganteus* [23]. K_m and V_{max} values were estimated using oat spelt xylan - a ramified arabinoxylan, and birchwood xylan - a xylan with few ramifications with

approximately 94% of xylose [42]. The purified xylanase exhibited typical Michaelis-Menten kinetics for both substrates, allowing the corresponding kinetic constants to be calculated. Xylanase I showed K_m values of 6.5 and 2.6 mg mL^{-1} and V_{max} values of 189.70 and 241.25 μmol min^{-1} mg^{-1} of protein, for birchwood and oat spelt xylans, respectively. Xylanase II exhibited K_m of 26.61 mg mL^{-1} with a V_{max} of 90.25 μmol min^{-1} mg^{-1} of protein for birchwood xylan and K_m of 23.45 mg mL^{-1} and V_{max} of 123.68 μmol min^{-1} mg^{-1} of protein, for oat spelt xylan. The K_m and V_{max} values exhibited by xylanases I and II are in agreement with the values presented by other fungal xylanases which range from 0.09 to 40.9 mg mL^{-1} for K_m and from 0.106 to 10.000 for V_{max} [4]. The values of K_m for these two substrates indicated that both xylanases had greater affinity for the oat spelt xylan, but higher affinity for this substrate is shown by xylanase I than xylanase II. Both xylanases also showed highest V_{max} values for oat spelt xylan. Thus, both purified xylanases have higher catalytic efficiencies for hydrolyzing oat spelt xylan. Similarly, the xylanase II from *A. giganteus* [23] exhibited higher affinity for oat spelt xylan and xylanase from *Fusarium oxysporum* [43] and xylanase III from *Acrophialophora nainiana* [44] showed highest value of V_{max} for oat spelt xylan.

Mode of action

The hydrolysis products of the purified xylanases were studied by ascending TLC analysis in reaction mixture incubated for period up to 17 hours (Fig. 8). The mobility of hydrolysis products in relation to xylose was similar to that described by Fontana et al. [19], using the same solvent system, revealing xylotriose, xylotetraose and larger xylooligosacharides formation. Xylanase I released xylobiose and other larger xylooligosaccharides, while xylanase II apparently only liberated xylooligosaccharides decreasing in polymerization degree up to xylotriose. Xylotriose is the smallest oligomer produced by most of the known xylanases [2]. Hence, these enzymes may be classified as endoxylanases.

Potential biotechnological applications

The purification and characterization of xylanolytic system from *P. sclerotiorum* indicate that xylanase I is a novel enzyme with interesting biochemical properties that make it a potentially candidate for industrial and commercial application in feed and food industries. Of particular interest is the fact that it shows an unusual optimum pH at 2.5 and remarkable

stability at acid conditions. One of the specific applications is in animal feed industry. Several studies demonstrated that the incorporation of xylanase into diet of animals results in the reduction of intestinal viscosity, thereby improving both weight gain and increasing feed conversion [45]. Xylanases with low pH optimum and high pH stability in acid pH would be most suitable for animal feed, due to extreme pH prevalence in the digestive tract. Through the application of acid stable enzymes, the nutrients availability in ruminant diets can be increase [46]. Most of the commercially available enzymatic products that have been tested as food additives for ruminants were not designed specifically for this purpose; enzymatic preparations containing cellulases and xylanases destined for use in the food, pulp, paper, textile, fuel and other chemical industries have been used [47]. Despite xylanase I exhibits lower levels of activity at animal's regular temperature, its combination with the substrate can significantly increase its performance. In addition, an increase of activity and thermal stability in this temperature can be achieved with directed-site mutagenesis. Thermal stability is the most commonly addressed stability parameter, whereas the structural basis of extreme pH stability is less well understood. Extreme pH activity and stability is also more difficult to be improved [48]. Specific applications for *P. sclerotiorum* xylanase I also include its use in clarification and maceration of juices and wines [49]. The extremely acid pH of must and wines limited the use of the enzymes in this process [50]. For a practical purpose it may be advantageous and efficient to utilize the acid xylanase obtained from this organism under harsh conditions in feed and food industries.

In addition, the other attractive biochemical characteristic which can be further explored is the xylobiose release through xylanase I action on xylan. For food applications, xylobiose and xylooligosaccharides have been used as a food ingredient to modulate the intestinal function since they could selectively be used by the beneficial gastrointestinal microbiota and suppress the growth of pathogenic bacteria [51]. The production of xylobiose is a time-consuming and expensive process [52]. The synthesis of xylooligosaccharides using hydrolytic enzymes such as xylanases is receiving great attention [53-55] and emerges as a great alternative to obtain these products.

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TABLES

Table 1 Purification of xylanases I and II from *P.sclerotiorum*.

Purification step		Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate		8551.7	85.40	100.14	1.00	100.0
DEAE-Sephadex A-50	Xyl I	5.454.9	36.13	150.98	1.51	63.8
	Xyl II	2075.6	48.65	42.66	0.43	24.3
Sephadex G-75	Xyl I	2318.7	9.30	249.15	2.49	27.1
	Xyl II	838.3	3.48	240.89	2.41	9.8

Table 02 Effect of different substances on relative activity of purified xylanases from *P. sclerotiorum*.

Substance	Xyl I activity (%)		Xyl II activity (%)	
	Concentration			
	2 mM	10 mM	2 mM	10 mM
Control	100	100	100	100
$CuCl_2$	89.8 ± 2.1	22.5 ± 1.7	49.7 ± 1.4	21.9 ± 0.5
$ZnSO_4$	82.1 ± 0.9	65.6 ± 1.7	85.7 ± 1.9	63.7 ± 1.9
$MnSO_4$	97.9 ± 1.4	99.3 ± 1.5	220.3 ± 3.6	225.8 ± 2.9
$BaCl_2$	110.2 ± 0.8	116.3 ± 1.3	96.0 ± 1.2	94.6 ± 1.1
$CaCl_2$	127.3 ± 1.2	128.7 ± 0.6	110.7 ± 1.7	122.0 ± 1.4
NH_4Cl	127.9 ± 1.2	130.0 ± 1.4	80.7 ± 1.8	75.2 ± 1.6
NaCl	99.7 ± 1.4	99.6 ± 0.8	102.5 ± 1.3	101.3 ± 1.7
SDS	ND	ND	13.9 ± 0.3	ND
PMSF	114.6 ± 0.7	118.6 ± 1.5	70.4 ± 1.5	64.2 ± 0.9
$MgSO_4$	95.5 ± 2.1	93.2 ± 1.5	98.6 ± 1.3	100.6 ± 1.4
Sodium citrate	108.5 ± 1.3	124.5 ± 2.3	111.6 ± 2.1	118.0 ± 1.1
DTT	141.7 ± 3.2	193.8 ± 3.4	148.4 ± 2.4	160.2 ± 3.8
$CoCl_2$	102.7 ± 1.2	103.3 ± 1.5	42.7 ± 1.2	ND
$HgCl_2$	50.7 ± 0.9	ND	63.9 ± 0.9	ND
$Pb(CH_3COO)_2$	99.6 ± 1.1	76.3 ± 2.1	99.2 ± 1.2	112.8 ± 0.9
EDTA	94.8 ± 0.9	63.0 ± 1.0	80.7 ± 1.1	27.9 ± 0.5
β-mercaptoethanol	143.1 ± 3.1	180.3 ± 2.8	109.6 ± 2.1	128.7 ± 2.3

FIGURES

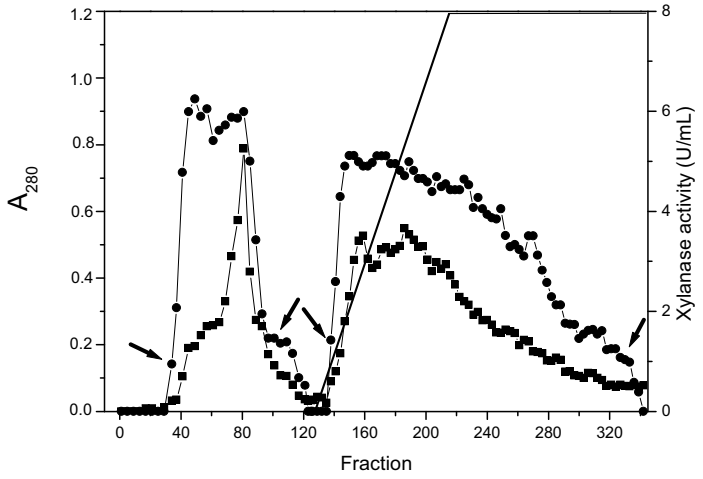

Fig. 1 DEAE-Sephadex A-50 chromatography of the xylanases from *P. sclerotiorum*. The arrows indicate the fractions pooled with xylanase activity. The column was equilibrated with 50 mM Tris-HCl buffer pH 9.0 and eluted with a linear salt gradient from 0.0 to 0.5 M in the same buffer. The flow rate and fraction size were 60 mL/h and 3.0 mL, respectively. (■) A_{280}; (●) xylanase activity and (–) NaCl concentration.

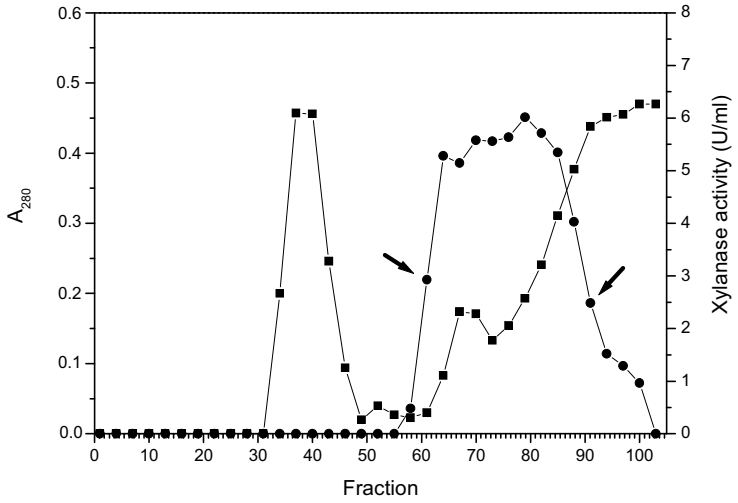

Fig. 2 Gel filtration on Sephadex G-75 of the xylanase I from *P. sclerotiorum*. The arrows indicate the fractions pooled with xylanase activity. The column was equilibrated and eluted with 50 mM ammonium acetate buffer pH 6.8. The flow rate and fraction size were 18 mL/h and 3.0 mL, respectively. (■) A_{280} and (●) xylanase activity.

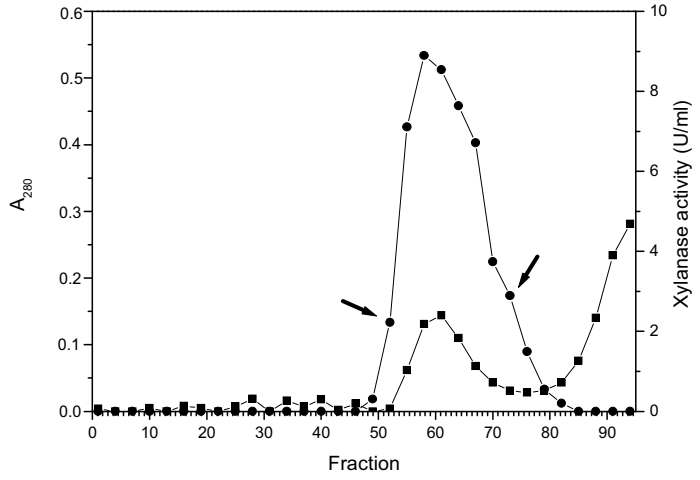

Fig. 3 Gel filtration on Sephadex G-75 of the xylanase II from *P. sclerotiorum*. The arrows indicate the fractions pooled with xylanase activity. The column was equilibrated and eluted with 50 mM ammonium acetate buffer pH 6.8. The flow rate and fraction size were 18 mL/h and 3.0 mL, respectively. (■) A_{280} and (●) xylanase activity.

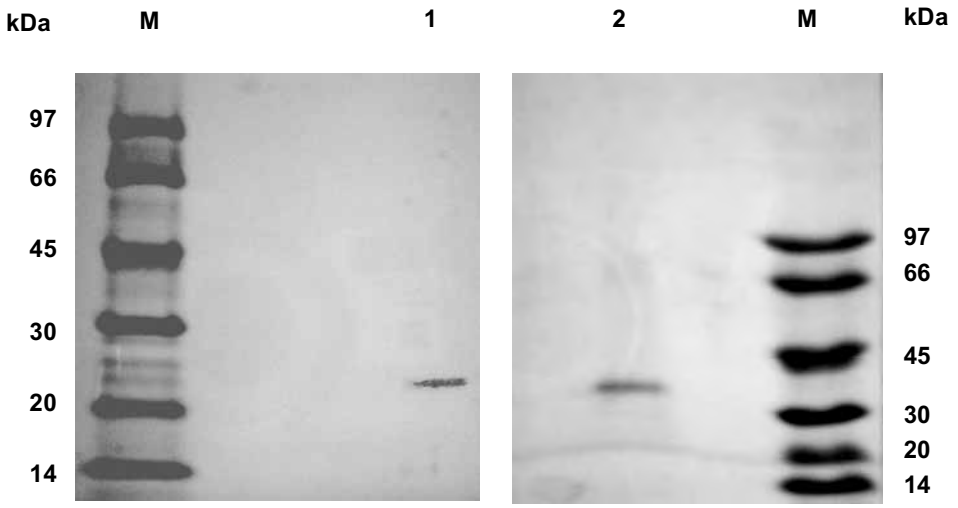

Fig. 4 SDS-PAGE (8-18%) of purified xylanase I and II from *P. sclerotiorum*. Lanes M: low molecular weight standard proteins; lane 1: xylanase I; lane 2: xylanase II.

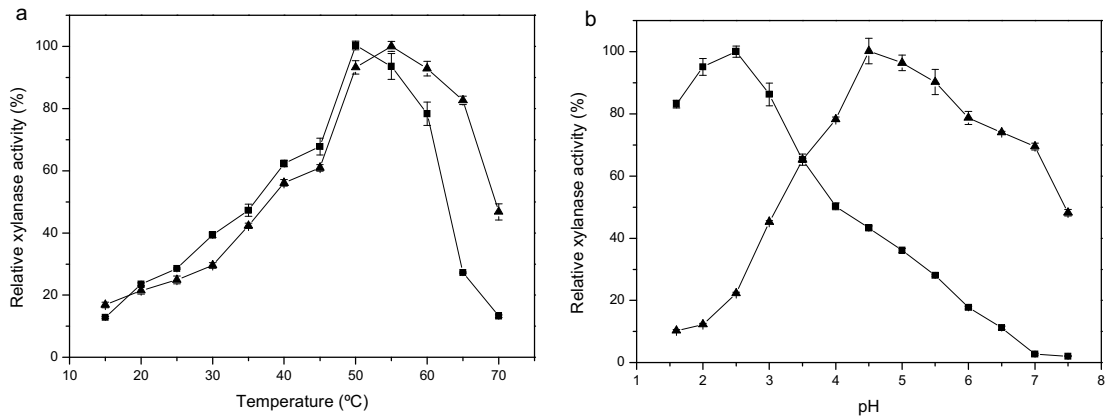

Fig. 5 Influence of temperature (a) and pH (b) on the xylanases I and II activities from *P. sclerotiorum*. Assay conditions: McIlvaine buffer pH 4.5 (a); 0.05 M glycine-HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer from 3.0 to 7.5; 50 ºC to xylanase I and at 55 ºC to xylanase II (b). Xylanase I (■) and xylanase II (▲).

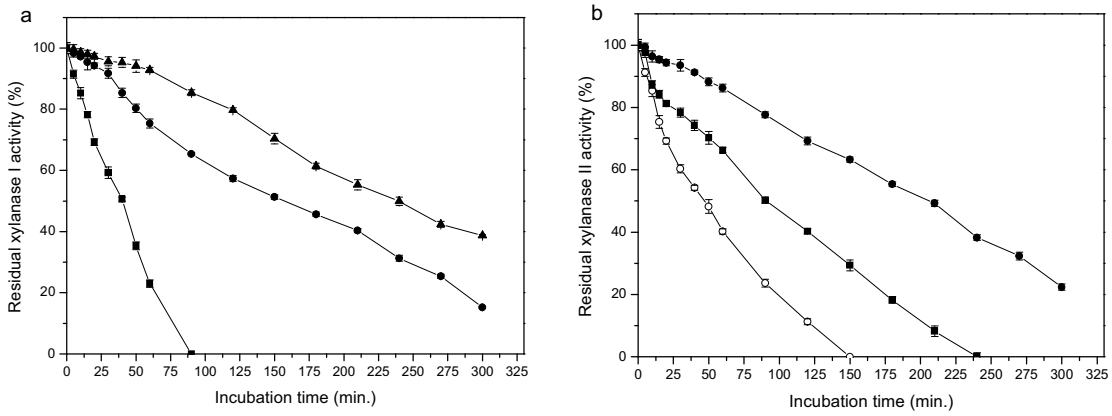

Fig. 6 Thermal stability of xylanase I (a) and xylanase II (b) activities from *P. sclerotiorum*. The purified xylanases were incubated at 40 (▲), 45 (●), 50 (■) and 55 °C (○) without substrate. Assay conditions: 0.05 M glycine-HCl buffer pH 2.5 (a) and McIlvaine buffer pH 4.5 (b).

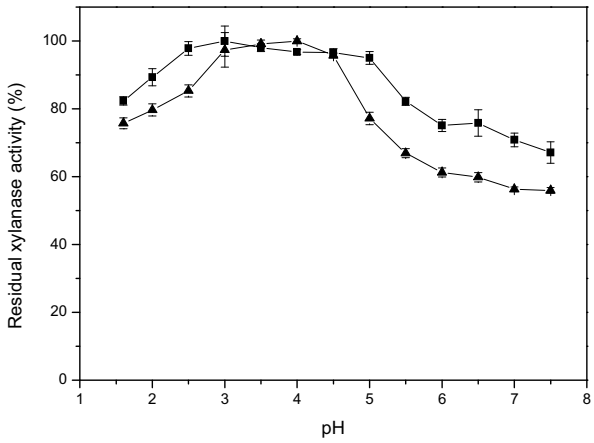

Fig. 7 pH stability of xylanase I (■) and xylanase II (▲) from *P. sclerotiorum*. The purified enzymes were pre-incubated without substrate with 0.05 M glycine-HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer from pH 3.0 to 7.5, at 4 ºC for 24 h.

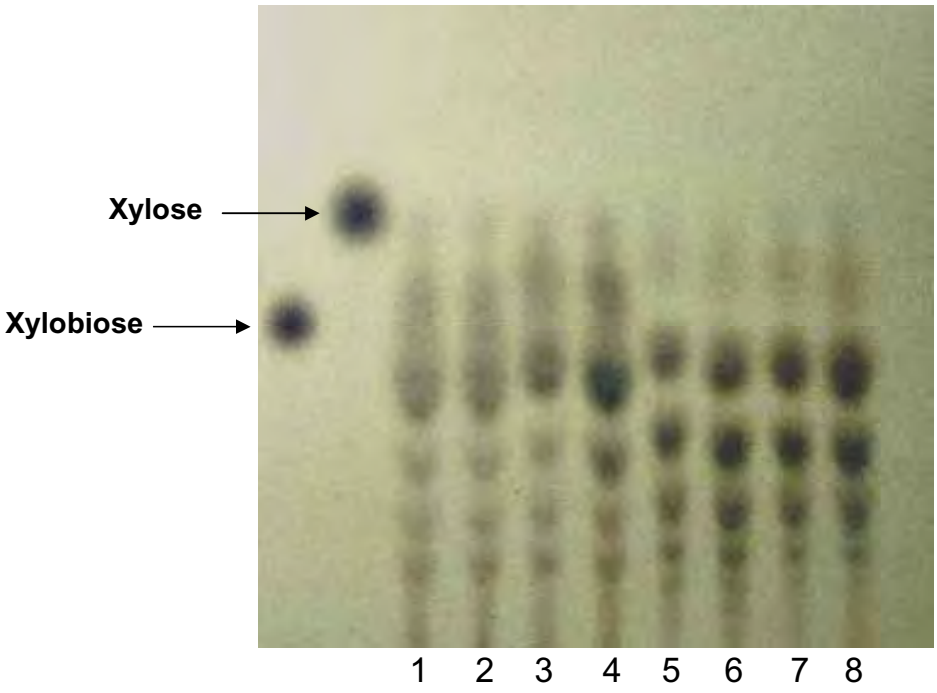

Fig. 8 Thin-layer chromatography of the hydrolysis products of oat spetl xylan from xylanases I and II. Lanes 1, 2, 3 e 4 represent hydrolysis products of xylanase I after 10, 30, 120 and 1020 min of incubation, respectively; lanes 5, 6, 7 e 8 represent hydrolysis products of xylanase II after 10, 30, 120 and 1020 min of incubation, in that order.

9. CAPÍTULO 5:

Purification and properties of a novel acid cell-associated β-xylosidase from *Penicillium sclerotiorum*

KNOB, A.; CARMONA, E.C. **Journal of Industrial Microbiology and Biotechnology** (artigo submetido).

Abstract

The β-xylosidase from *P. sclerotiorum* was purified to homogeneity by a rapid procedure, using ammonium sulfate fractionation and molecular exclusion chromatography. SDS-PAGE analysis reveled two bands with estimated molecular mass of 97 and 42 kDa. In non-denaturing PAGE**,** eletrophoretical homogeneity was observed. These results indicate that this protein shows dimeric structure. The molecular mass of native enzyme estimated by molecular exclusion chromatography was 144 kDa. The enzyme is a glycoprotein with 56.4% carbohydrate content. The pH and temperature optima were 2.5 and 60 ºC, respectively. The enzyme remained stable over a pH range from 2.0 to 7.0 and up to 60 ºC for 375 min. All divalent cations tested, except Hg^{2+}, inhibited β-xylosidase activity, especially at 10 mM concentration. The purified enzyme was also sensitive to denaturing agents SDS, EDTA and activated by thiol-containing reducing agents. The Michaelis-Menten constant for p-nitrophenyl-β-D-xyloside was 0.78 mM and the maximum reaction velocity was 0.51 μmole PNXP min^{-1} mg^{-1} of protein. This is the first report about purification and characterization of a β-xylosidase from *P. sclerotiorum*, which presented potential application in some biotechnological process such as animal feed, juice and wine industries.

Key words: *Penicillium sclerotiorum*; β-xylosidase; enzyme purification; enzyme characterization

Introduction

Xylan is the major constituent of hemicellulosic polysaccharide in cell walls of land plants, representing up to 30-35% of the total dry weight [3]. The backbone of xylan is composed of β-1,4-linked D-xylopyranosyl residues. Most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone chain, such as acetyl, 4-*0*-methyl-D-glucuronosyl or L-arabinosyl groups [5, 44].

The structure of xylan varies depending on its origin, so it is not surprising that complete hydrolysis of these complex molecule requires the synergistic action of several microbial enzymes. The most important xylanolytic enzymes are endo-β-1,4-xylanase (EC 3.2.1.8), which hydrolyses the insoluble xylan backbone in shorter, soluble xylo-oligosaccharides, and β-xylosidase (EC 3.2.1.37), which hydrolyses the soluble xylooligosacharides and xylobiose from the nonreducing end to liberate xylose [40].

Interest in xylan-degrading enzymes has greatly increased in the last decade due to their potential biotechnological applications, especially in the development of environmentally friendly technologies in the food, pulp and paper industries and bioconversion [6, 32, 40]. Development of biotechnological methods from xylan for polymer conversion via xylose into acetate, propionate, lactate, ethanol, xylitol and other commercial value compounds [16, 21, 30] by using microbial fermentation technologies has increased the β-xylosidase application by chemical and pharmaceutical industries in recent years.

β-Xilosidases have been described from a variety of microorganisms. However, the majority of studies has focused on xylanases [26, 40]. Filamentous fungi are widely used as β-xylosidase producers and are generally considered as more potent xylanolytic enzymes producers than bacteria and yeasts [32]. Some fungal β-xylosidases, including *Penicillium* and *Aspergillus* species have been purified and biochemically characterized [33, 35] but, in most of cases, the purification procedure is laborious. A strain of the fungus *P. sclerotiorum* produces high levels of cell-associated β-xylosidase activity when grown on oat spelt xylan as carbon source [20]. In this paper, we report a rapid and efficient purification procedure of the β-xylosidase from *P. sclerotiorum*; its biochemical and catalytic properties are described and possible biotechnological applications are presented.

Materials and methods

Organism and growth

P. sclerotiorum, from the culture collection of Environmental Studies Center - CEA/UNESP, SP, Brazil, was isolated from soil of the Atlantic Forest, Brazil. Conidia were obtained from Vogel solid medium cultures [41] containing 1.5% (w/v) glucose and 1.5% (m/v) agar at 25 ºC for 7 days. Liquid cultures were prepared in the same medium (pH adjusted to 5.0) with 1% (w/v) oat spelt xylan as carbon source. Erlenmeyer flasks (125 ml) containing 25 ml of medium were inoculated with 1.0 ml of spore suspension (5 x 10^7 spores/ml) and incubated at 30 ºC for 6 days in a stationary condition.

Enzyme preparations and assays

The mycelium was removed by vacuum filtration and it was used as crude enzyme extract. The same was washed with distilled and sterilized water, frozen and ground with sand in 50 mM McIlvaine buffer pH 4.0. The suspension was centrifuged at 3.900 *g* at 4 °C and the supernatant was used as a cell-associated enzyme source. β-xylosidase activity was determined according to Kersters-Hilderson et al. [18] measuring the p-nitrophenol released from p-nitrophenyl β-D-xylanopiranoside (PNPX) at 405 nm. A 0.2 ml solution of 0.25% PNPX (w/v) was preincubated for 5 min in 0.05M glycine-HCl buffer, pH 2.5, at 60 °C. To this solution, 0.3 ml of the properly diluted enzyme sample was added. After an appropriate period, aliquots of 0.1 ml were retired and the reaction was stooped by addition of 1.0 ml of saturated sodium tetraborate solution. One unit of enzyme activity was defined as the enzyme amount that releases 1 μmol of p-nitrophenol per ml, per min of reaction. Specific activity was expressed as unit per milligram of protein. All the experimental data presented here are mean values of three independent measurements standard deviations (SD).

Protein determination

Protein content was determined by the Lowry method [27] with bovine serum albumin as standard, or by monitoring the u.v. absorbance at 280 nm.

Purification of acid cell-associated β-xylosidase

Ammonium sulfate fractionation

The crude enzyme (50 ml) was fractionated by ammonium sulfate precipitation (0-60%, w/v). The supernatant of 60% ammonium sulfate ***saturation*** obtained after centrifugation (6.000g, 20 min) contained the highest enzyme proportion. This sample was extensively dialyzed against 0.05 M ammonium acetate buffer pH 6.8, lyophilized and dissolved in minimum volume of the same buffer.

Molecular exclusion chromatography

The protein sample obtained in the step above was chromatographed on Sephadex G-100 column (2.6 x 64.0 cm) equilibrated and eluted with 0.05 M ammonium acetate buffer, pH 6.8, flowing at 18 ml/h. Fractions (3 ml) were collected whose protein content was estimated by reading absorbance at 280 nm and β-xylosidase activity assayed as described previously.

Electrophoresis

Polyacrylamide gels were prepared according to Laemmli [23]. SDS-PAGE was carried out in an acrylamide 12.5% (w/v). Native PAGE gel (10.0% acrylamide, w/v) was performed in the absence of sodium dodecyl sulfate (SDS). Samples were mixed with an equal volume of sample buffer that did not contain SDS or β-mercaptoethanol. Proteins were stained with 0.1% (w/v) Coomassie brilliant blue R-250 diluted in methanol-acetic acid-water (5:2:5, v/v/v). The proteins phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, tripsin inhibitor and α-lactalbumin were used to construct the standard curve of log molecular weight against relative mobility in the gel.

Purified β-xylosidase characterization

Determination of carbohydrate concentration

Total carbohydrate was measured colorimetrically by the Dubois phenol-sulphuric acid method [10], with glucose as standard.

Optimum pH and temperature β-xylosidase activity

The purified β-xylosidase activity was measured at 60 ºC in different pH values using 0.05 M glycine-HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer from 3 to 7.5. The optimum temperature was performing the reaction in a temperature range between 20 and 85 ºC in the optimum pH.

β-xylosidase stability at different temperature and pH

For pH stability assay, the enzyme preparation was diluted (1:2) in 0.05 M glycine-HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer in a pH range from 3.0 to 7.5. The samples were incubated at 4 ºC for 24 h. After this period, the β-xylosidase activity was assayed in optimal conditions. To evaluate the thermal stability, the enzyme preparation was incubated at 60, 65 and 70 °C at optimum pH determined above for different periods. The residual activity was determined in each sample, at the optima pH and temperature.

Effect of substances on β-xylosidase activity

The β−xylosidase was tested against a wide array of metallic ions and other compounds at final concentrations of 2 mM and 10 mM and the activity was measured by performing the enzyme assay in optimal conditions.

Kinetic parameters

The purified β-xylosidase was incubated with PNPX, at concentrations between 0.5 and 15 mM at pH 2.5 and 60 ºC for 5 min and 10 min. The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated from Lineweaver-Burk reciprocal plots, using ‘GraFit’ 5.0 software.

Specificity of purified β-xylosidase

Activities towards p-nitrophenyl derivatives were measured by the rate of p-nitrophenol formed during hydrolysis from 5 mM of the substrates in 0.05 M glycine-HCl buffer pH 2.5, at 60 ºC for 5 and 10 min and detected by spectrophotometry, at 405 nm.

Tolerance of purified β-xylosidase to xylose

To verified the xylose inhibition effect, the enzymatic assay was performed by incubating 10 µl purified enzyme, 200 µl of 1mM PNPX dissolved in 0.05 M glycine-HCl buffer pH 2.5 and 300µl of xylose in varying dilutions at final concentration of 5 to 100 mM, at 60 ºC for 5 min and 10 min. The K_i for this inhibitor was calculated using ´GraFit' 5.0 software.

Results and Discussion

Purification of cell-associated β-xylosidase

Conventional purification methods were effective in isolating and purifying the *P. sclerotiorum* β-xylosidase. This enzyme was purified from the cell-associated preparation by protein precipitation with ammonium sulfate and molecular exclusion chromatography. The molecular exclusion chromatography elution profile resulted in one peak of β-xylosidase activity (Fig. 1). The fractions corresponding to this peak were pooled and the sample was submitted to electrophoretic analysis. The purification of the β-xylosidase is summarized in Table 1. The procedure resulted in an overall yield of 46.07 % and the specific activity increased 2.42 fold. The purified β-xylosidase exhibited high specific activity corresponding to 31.12 U mg^{-1} protein. In comparison, other preparations of purified fungal β-xylosidases showed low specific activities. β-Xylosidase from *Humicula grisea* var. *thermoidea* presented 19.60 U mg^{-1} protein [7], whereas β-xylosidases from *Cochliobolus carbonum* [34], *Aspergillus awamori* [11] and *Trichoderma harzianum* [46] exhibited specific activities of 7.4, 4.2 and 3.42 U mg^{-1} protein, respectively.

SDS-PAGE revealed a double protein band with molecular weight estimated according to their apparent mobility of 97 and 42 kDa. The molecular mass of the native enzyme estimated by molecular exclusion chromatography was 144 kDa (Fig. 02). The enzyme purity was confirmed by PAGE under nondenaturing conditions, which showed electrophoretic homogeneity (Fig. 02). Dimeric forms are widespread among β-xylosidases purified from filamentous fungi [11, 22, 25, 39]. The carbohydrate content of purified enzyme was estimated to be 56.4 %. High carbohydrate content was also verified for β-xylosidase from *Aspergillus versicolor* (100 kDa - 47%) [2], *Paecilomyces thermophila* (61.5%) [42] and *Aspergillus phoenicis* (43.5%) [35]. However, this carbohydrate content is much higher than those of the β-xylosidase from *Scytalidium thermophilum* (12%) [47] and another from *A. versicolor* (60 kDa – 21%) [2].

Enzymatic properties of β-xylosidase

Effects of pH and temperature on the β-xylosidase activity

The β-xylosidase activity showed optimal activity at pH 2.5 (Fig. 3a). Only *Aspergillus pulverulentus* showed a similar optimum pH value between 2.5 and 3.5 [39] and the β-xylosidase produced by yeast *Aureobasidium* sp. showed optimum pH of 3.0 [24]. Generally, β-xylosidases of fungal origin show optimal activity at pH from 4.0 to 6.0 [14, 35, 37, 39, 43].

The purified enzyme is most active at 60 ºC (Fig. 3b), which is higher than that observed for the the β-xylosidases from *Penicillium herquei* (50 ºC) [15], *A. nidulans* (50 ºC) [22], *P. thermophila* (55 ºC) [42] and the thermophilic fungus *Sporotrichum thermophile* (50 ºC) [17]. Similarly, β-xylosidase from *Fusarium proliferatum* exhibited optimum temperature at 60 ºC [37]. *Fusarium verticillioides* β-xylosidase showed maximal activity at 65 ºC [36].

3.2.2 Effects of pH and temperature on the stability of the β-xylosidase

Thermal and pH stability of β-xylosidase were investigated (Fig. 4). The purified β-xylosidase presented higher stability in its optimum pH and remains stable with stability above 70% in all acid and neutral conditions evaluated. The only exception was pH 7.5, in which less than 60% of activity was verified.

To verify the enzyme stability at different temperatures, the purified enzyme was incubated without substrate at 60, 65 and 70 ºC. This enzyme was stable at 65 ºC, retaining 75% of its activity over 90 min at this temperature. High thermal stability was verified at 60 ºC, with half life-time of 375 min. Studies of thermostability indicated that purified β-xylosidase is more thermostable than many fungal β-xylosidases, such as those obtained from *Aspergillus carbonarius* [19], *A. versicolor* [2] and *Talaromyces thermophilus* [13]. This is an advantageous property of *P. sclerotiorum* β-xylosidase, since in the most industrial process where xylanolytic enzymes can be applied are carried out at high temperatures [45].

Effect of substances on purified β-xylosidase activity

In order, to verify the effect of substances on β-xylosidase activity, the purified enzyme was assayed in presence of several metallic ions, sodium dodecylsufate (SDS), tetrasodium ethylenediaminetetraacetatic acid (EDTA), phenilmethylsulphonyl fluoride (PMSF), dithiotreitol (DTT) and β-mercaptoethanol, at 2 mM and 10 mM concentrations (Table 2). β-Xylosidase was strongly inhibited by all divalent cations tested, especially at 10 mM concentrations, except Hg^{2+}, which did not affect β-xylosidase activity. The same effect was verified to *T. harzianum* [46] and *Trichoderma koningii* [25] β-xylosidases. However, in most cases, Hg^{2+} acts as strong inhibitor of xylan-degrading enzymes. Strong inhibition by heavy-metal ions as Pb^{2+} and Mn^{2+} is a common feature of β-xylosidases from fungi [29].

The monovalent cation Na^{+} also inhibited β-xylosidase activity, especially at 10 mM. The β-xylosidase was significantly activated by citrate and NH_4^{+} at 10 mM concentration, suggesting that NH_4^{+} is a cofactor required for this enzyme. Here, the citrate stimulation observed can be due to this action as chelant, which posses a pronounced selectivity for Mn^{2+}, Mg^{2+} and other ions of similar size [12] removing ions that had an adverse effect on β-xylosidase activity.

Conversely to citrate, EDTA, other divalent cation chelanting agent assayed, negatively influenced β-xylosidase activity. In this case, it seems that some ion, which is not affected by citrate but exclusively by EDTA and whose influence was not evaluated in this study, can also be required for enzyme action. Total loss of activity was observed in the presence of SDS, indicating that hydrophobic interactions must be important in maintaining β-xylosidase structure. The reducing agents β-mercaptoethanol and DTT stimulated β-xylosidase activity, suggesting an influence of cysteine thiol groups in the catalysis of PNPX.

Enzyme kinetic investigations

The rate dependence of the enzymatic reaction on the PNPX concentration at pH 2.5 and 60 ºC followed Michaelis-Menten Kinetics (data not shown). The apparent V_{max} and K_m values for the purified β-xylosidase determined by Lineweaver-Burk plots was 0.51 μmole PNXP min^{-1} mg^{-1} of protein and 0.78 mM, respectively. These values are very similar to the ones obtained for purified *F. proliferatum* β-xylosidase [37] and they are in accordance with the published values for other fungal purified β-xylosidases. *P. sclerotiorum* β-xylosidase showed greater substrate affinity when compared to *S. thermophilum* [47], *S. thermophile* [17], *P. thermophila* [42] and *T. thermophilus* [13] β-xylosidases. Lower K_m values than this were reported to purified enzymes from *Neurospora crassa* [9] and *A. versicolor* [2].

Substrate specificity

The purified enzyme showed arabinofuranosidase activity, corresponding to only 3 % of PNPX hydrolysis, but the enzyme did not hydrolyze PNP-Gal and PNP-GP substrates. α-L-arabinosidase activity was also described for many fungal β-xylosidases including β-xylosidases from *Penicillium wortmanni* [8], *A. carbonarius* [19] and *P. thermophila* [42]. The observed dual β-xylosidase and arabinofuranosidase of the purified enzyme is interesting for substituted xylans utilization. The spatial similarity between D-xylopyranose and L-arabinofuranose can be responsible for bifunctional xylosidase-arabinofuranosidase enzymes existence [25, 28]. The purified *P. sclerotiorum* β-xylosidase is free from xylanase activity, since the purified enzyme did not degrade polymeric xylans such as oat spelt xylan and birchwood xylan. In contrast, *Trichoderma reesei* β-xylosidase is a multifunctional β-D-xylan xylohydrolase, which posses α-L-arabinofuranosidase activity and remarkable ability to release xylose from xylan [14].

Inhibition by xylose

D-xylose inhibited β-xylosidase activity, with K_i of 32.1 mM (data not shown). As *P. sclerotiorum* β-xylosidase, several other β-xylosidases have proved to be xylose tolerant. Similarly, *A. nidulans* β-xylosidase was inhibited in the presence of xylose at 25 mM [22]

while β-xylosidase from *P. thermophila* was competitively inhibited by xylose with a K_i value of 139 mM [42]. However, the majority of β-xylosidases presented a K_i for xylose ranging from 2 to 10 mM [14, 19, 36, 37]. The xylose tolerance is a desirable feature for β-xylosidase application in xylan saccharification process [47].

Potential biotechnological applications

The purified β-xylosidase demonstrated optimum activity and stability at very acid pH and 60 ºC. This information is important, because it means that this enzyme shows valuable characteristics for industrial purpose. β-Xylosidases have been used in enological applications for releasing glycosides from monoterpenes [38]. The action and high stability of purified β-xylosidase in acidic pH make its interesting for application in this process as well as in clarification and maceration of juices and wines, where enzymes must act at very acidic conditions [1]. The β-xylosidase characteristics also make it interesting for application in animal feed industry. Many studies have proved that the addition of β-xylosidases to diets improves nutritive values of feed and animal performance [4, 31]. Its optimum pH of activity is consistent with the extreme pH prevalent in the animal digestive tract. Moreover, given its activity in low pH and remarkable stability at 60 ºC, it is possible to prevent the undesirable growth of microorganisms contaminants in industrial processes, giving additional advantage to the industrial application of this novel enzyme.

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TABLES

Table 1. Purification of β-xylosidase from *P. Sclerotiorum*.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate	171.55	13.38	12.82	1.0	100.00
Supernatant 60% of $(NH_4)_2SO_4$	162.49	5.30	30.66	2.39	94.72
Sephadex G-100	79.04	2.54	31.12	2.42	46.07

Table 02. Effect of different substances on relative activity of β-xylosidase from *P. sclerotiorum*.

Relative activity (%)		
Substance	Concentration	
	2 mM	10 mM
Control	100.0	100.0
$CuCl_2$	67.7 ± 1.2	26.5 ± 0.6
$ZnSO_4$	86.5 ± 1.6	33.7 ± 2.3
$MnSO_4$	37.9 ± 2.1	34.1 ± 2.2
$BaCl_2$	75.9 ±1.0	56.9 ± 0.8
$CaCl_2$	74.1 ± 1.7	36.6 ± 2.3
NH_4Cl	100.1 ± 0.5	125.6 ± 2.5
NaCl	81.0 ± 3.4	23.7 ± 0.3
SDS	ND	ND
PMSF	94.8 ± 1.2	90.9 ± 0.6
$MgSO_4$	64.3 ± 2.6	22.6 ± 0.5
Sodium citrate	99.6 ± 2.7	138.8 ± 3.9
DTT	104.2 ± 1.2	114.4 ± 2.0
$CoCl_2$	80.7 ± 3.3	53.9 ± 2.9
$HgCl_2$	98.3 ±1.6	96.9 ± 2.6
$Pb(CH_3COO)_2$	75.9 ± 1.3	58.5 ± 2.2
EDTA	91.9 ± 1.0	62.9 ± 1.4
β-mercaptoethanol	136.3 ± 2.5	144.2 ± 2.4

FIGURES

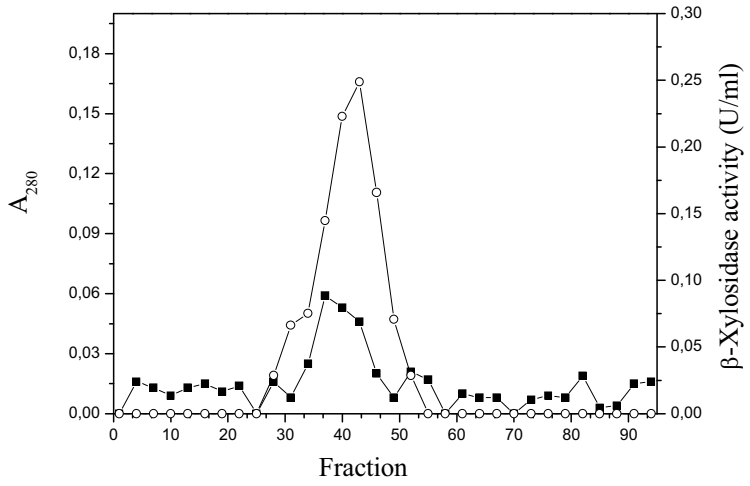

Fig. 1 Gel filtration on Sephadex G-100 of the β-xylosidase from *P. sclerotiorum*. Conditions: 50 mM ammonium acetate buffer pH 6.8; flow rate 18.5 ml/h, fraction size 3.0 ml. (■) A_{280} and (○) β-xylosidase activity.

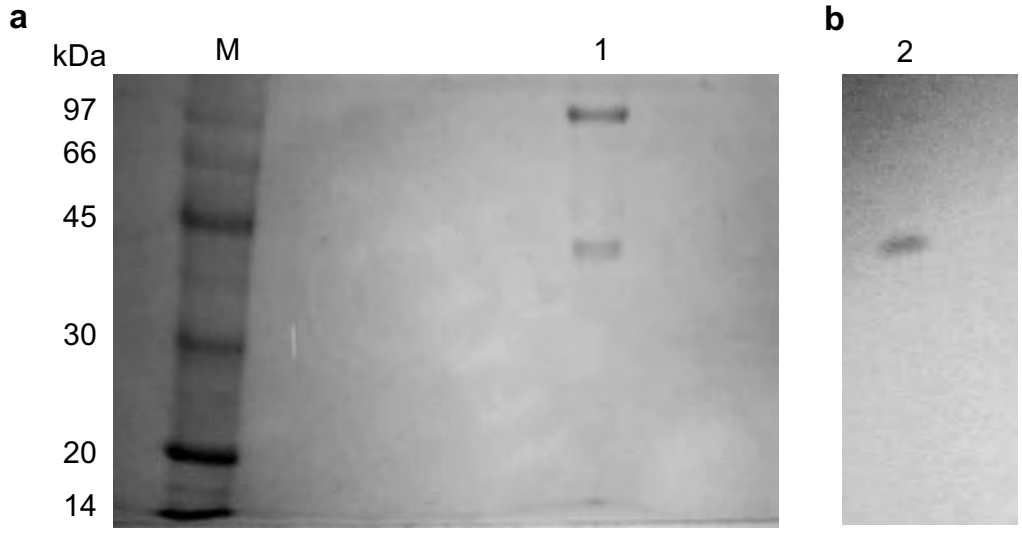

Fig. 2 SDS-PAGE (12.5%) (a) and native PAGE (10.0%) (b) of purified β-xylosidase from *Penicilium sclerotiorum*. Lane M: low molecular weight calibration kit; lane 1: fraction of Sephadex G-100 column; lane 2: PAGE of the purified β-xylosidase.

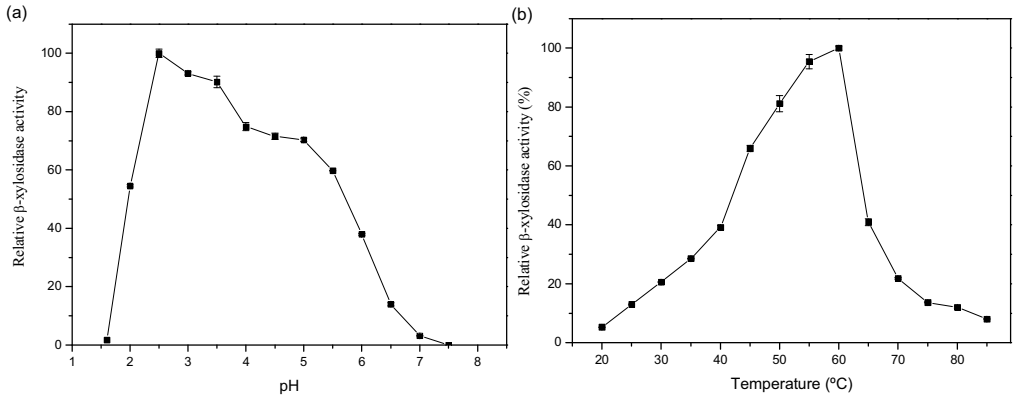

Fig. 3 Influence of pH (a) and temperature (b) on the β-xylosidase activity from *P. sclerotiorum*. Assay conditions: (a) 0.05 M glycine-HCl buffer for pH 1.6 to 2.5 and McIlvaine buffer from 3.0 to 7.5, at 60 ºC. (b) 0.05M glycine-HCl buffer pH 2.5.

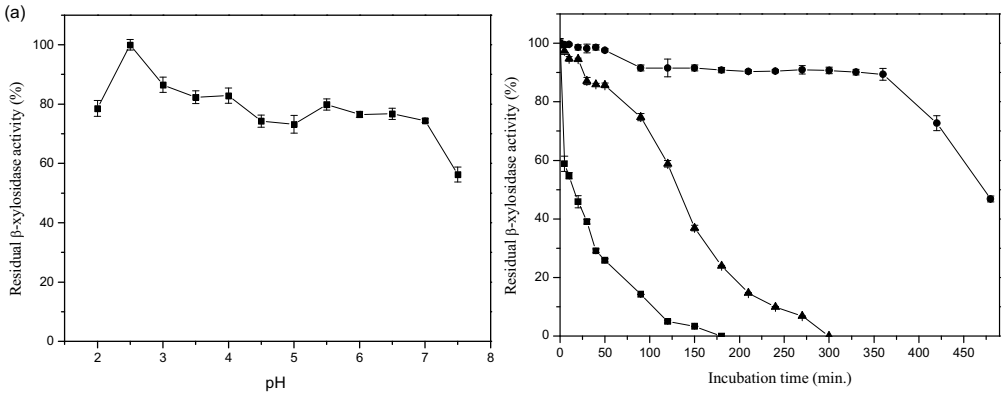

Fig. 4 pH (a) and thermal (b) stability of β-xylosidase activity from *P. sclerotiorum*. (a) The purified enzyme was incubated without substrate with 0.05 M glycine-HCl buffer pH 2.0 and 2.5 and McIlvaine buffer from pH 3.0 to 7.5, at 4 ºC for 24 h. (b) The purified enzyme was incubated at (●) 60, (▲) 65 and (■) 70 ºC without substrate. In both, the residual β-xylosidase activity was determined with 0.05 M glycine-HCl buffer, pH 2.5, at 60 ºC.

10. CONSIDERAÇÕES FINAIS

A eficiente produção de enzimas xilanolíticas por microrganismos depende da escolha do substrato, da otimização da composição do meio de cultura bem como da determinação das melhores condições de cultivo. Através da seleção destes, a produção de xilanases e β-xilosidases por *Penicillium sclerotiorum* foi aumentada em 3,5 e 4 vezes, respectivamente. A fonte de carbono que induziu maiores níveis de atividade xilanolítica foi a xilana de aveia, em cultivo estacionário. Maiores níveis de atividade xilanásica foram obtidos no 5º dia de cultivo, enquanto que maiores níveis de atividade β-xilosidásica foram obtidos no 6º dia de cultivo, um dia logo após a produção máxima de xilanases. O pH e a temperatura de cultivo estabelecidos para a máxima produção de xilanases foram pH 6,5 e 30 ºC, e para a produção de β-xilosidases associadas às células foram pH 5,0 e 30 ºC.

As enzimas xilanolíticas presentes em suas preparações brutas foram caracterizadas bioquimicamente. A atividade xilanásica do filtrado de cultura apresentou temperatura ótima de 50 ºC, pH ótimo de 4,5 e considerável estabilidade térmica a 40 ºC e em pHs entre 3,5 e 7,5. A atividade β-xilosidásica, por sua vez, apresentou temperatura ótima de 60 ºC, pH ótimo de atividade 2,5 e permaneceu relativamente estável a 50 e 55 ºC e em pH variando de 2,0 a 4,0 e 7,5.

Como os processos de purificação correspondem a uma importante parcela nos gastos de produção, a seleção de técnicas apropriadas que maximizem a recuperação e o grau de purificação da enzima e que envolvam menor número de etapas faz-se necessária. Neste trabalho, através do emprego de técnicas convencionais, foi possível obter a purificação das enzimas xilanolíticas produzidas por *P. sclerotiorum*, de forma rápida e eficiente.

O sistema xilanolítico produzido por *P. sclerotiorum* nas condições otimizadas produziu duas endoxilanases, as quais foram denominadas xilanases I e II. As massas moleculares determinadas por filtração em gel e por PAGE-SDS corresponderam, respectivamente, a 23,9 e 23,8 kDa para a xilanase I e 33,1 e 30,8 kDa para a xilanase II. Ambas as enzimas são glicoproteínas, sendo os valores de pH e temperatura ótimos, 2,5 e 50 °C para xilanase I, e 4,5 e 55 °C para xilanase II. As enzimas purificadas apresentaram-se mais estáveis termicamente quando comparadas as presentes no extrato bruto. A 50 ºC, a preparação enzimática bruta apresentou meia-vida de apenas 4 min, enquanto que as meias-vidas para as xilanases I e II purificadas foram de 40 e 90 min, respectivamente. A faixa de pH entre 3,5 e 7,5 na qual as enzimas presentes na preparação bruta mostraram-se estáveis foi

similar à exibida pela xilanase I. Porém, a xilanase II purificada apresentou uma faixa mais estreita de estabilidade, entre os pH 2,5 e 4,5. Em relação ao efeito de substâncias, Hg^{+2}, Cu^{+2} e SDS foram fortes inibidores enquanto que DTT e β-mercaptoetanol ativaram a atividade de ambas as enzimas purificadas. Além disso, o íon Mn^{+2} foi capaz de ativar a atividade da xilanase II.

Ambas as enzimas purificadas mostraram-se específicas para xilano, apresentando comportamento michaeliano típico e maior afinidade pela xilana de aveia em relação à xilana de birchwood. A hidrólise da xilana de aveia pela xilanase I liberou xilobiose e oligossacarídeos maiores, enquanto que apenas xilotriose e oligossacarídeos com um maior grau de polimerização foram liberados pela ação da xilanase II. A ação conjunta destas xilanases, com diferentes modos de ação, pode contribuir para uma hidrólise mais efetiva da xilana.

Apenas uma β-xilosidase associada às células de *P. sclerotiorum* foi detectada e purificada. Esta enzima é glicosilada, apresentando-se como um dímero. A análise em PAGE-SDS relevou duas bandas de proteínas, de massas moleculares de 97 e 42 kDa. Por meio da filtração em gel, a massa molecular da enzima nativa foi estimada em 144 kDa. Esta β-xilosidase apresentou atividade ótima em pH 2,5 e 60 ºC. Nota-se que essas mesmas propriedades já haviam sido determinadas no estrato bruto. Porém, quando esta enzima foi purificada, sua estabilidade térmica aumentou consideravelmente. Na preparação bruta, esta enzima apresentou meia-vida de apenas de 20 min a 60 ºC, passando a apresentar meia-vida de 375 min, nesta mesma temperatura, quando purificada. Esta também passou a ser mais estável em uma faixa mais ampla de pH em relação à enzima não purificada. Na preparação bruta, a enzima foi estável em pH variando de 2,0 a 4,0 e 7,5, enquanto que a enzima purificada foi estável em pH entre 2,0 e 7,0. A remoção de proteases presentes na preparação bruta pode ter contribuído para o aumento da estabilidade da enzima quando purificada. Não houve diferenças expressivas em relação ao efeito de íons sobre a atividade β-xilosidásica na preparação bruta e na forma purificada. Como verificado anteriormente, cátions divalentes inibiram a atividade β-xilosidásica, especialmente quando presentes na concentração de 10 mM. EDTA e SDS também foram inibidores desta atividade. Inversamente, DTT e β-mercaptoetanol ativaram a atividade β-xilosidásica. A enzima purificada apresentou baixos níveis de atividade α-L-arabinofuranosidásica e se mostrou tolerante a xilose, apresentando K_i de 32.1 mM.

Neste trabalho, *P. sclerotiorum* foi caracterizado como um excelente produtor de xilanases e de β-xilosidases. A purificação e a caracterização dos componentes de seu complexo xilanolítico contribuíram para o conhecimento desse sistema em uma espécie na qual ele não havia sido descrito. Estas enzimas apresentaram características interessantes para algumas aplicações industriais, nas quais é imprescindível o emprego de enzimas atuantes e estáveis em pH de elevada acidez, como nas indústrias de ração animal, de sucos e de vinhos.

Novos experimentos envolvendo a ação conjunta da β-xilosidase e das xilanases purificadas são sugeridos, a fim de ampliar as informações sobre o modo de ação e a eficiência catalítica destas enzimas, contribuindo, por sua vez, para elucidar o processo de degradação da xilana, de diferentes origens e sobre diferentes condições. Estudos envolvendo a imobilização destas enzimas também seriam interessantes, a fim de se obter enzimas termicamente mais estáveis.

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